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July 1971
Volume 215 Number 3

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The Journal of Physiology

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J. Physiol. (1971), **215**, *pp.* 577–590
With 10 *text-figures*
Printed in Great Britain

LOCAL BLOOD FLOW, CEREBROVASCULAR AUTOREGULATION AND CO_2 RESPONSIVENESS IN THE RABBIT HYPOTHALAMUS

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(*Received 9 March 1970*)

SUMMARY

1. Local blood flow has been measured in the hypothalamus and other areas of the brain in the conscious rabbit, by measuring the rate of clearance of small volumes (2·5–10 μl.) of a saline solution of the inert tracer ^{133}Xe injected locally.

2. Increasing the arterial P_{CO_2} caused a mean rise in the hypothalamic blood flow of 34%.

3. The hypothalamic blood flow remained relatively constant over a mean arterial blood pressure range of 41–140 mm Hg.

4. Hypothalamic blood flow was relatively unaffected by anaesthesia with intravenous pentobarbitone; by contrast, blood flow in the cerebral cortex was greatly reduced.

5. It is concluded that the technique is a valid and useful one, since CO_2 responsiveness and autoregulation is maintained. Different areas of grey matter have different flow rates, and vary in their responsiveness to barbiturate anaesthesia.

INTRODUCTION

Methods for the measurement of cerebral blood flow have evolved rapidly in the 25 years since Kety & Schmidt (1945) first described their nitrous oxide technique. An important development has been the measurement of 'compartmental' flow, i.e. flow in grey and white matter, from the clearance of radioactive inert gases, such as ^{85}Kr and ^{133}Xe, infused or injected up the carotid artery (Lassen *et al.* 1963). These gases equilibrate rapidly between blood and brain tissue, and when the injection is stopped the gas is cleared from the brain tissue by the perfusing blood. This rate of

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clearance can be measured by means of an external scintillation counter. The curve of disappearance of radioactivity, when plotted on semilogarithmic paper, can be analysed into two components, a fast and a slow component, corresponding to flow through grey and white matter respectively (Kety, 1965; Häggendal, Nillson & Norback, 1965).

A refinement of this approach is the use of multiple external collimated counters, each looking at a core of brain tissue; however, the localization is still relatively crude, and it is impossible to distinguish between cortical flow and non-cortical grey matter flow in the same counting field. Under certain circumstances this may be of importance, and this paper describes a technique for the measurement of local cerebral blood flow in the conscious animal, by a ^{133}Xe injection technique. Local injection methods have been described (Nillson, 1965; Espagno & Lazorthes, 1965; Häggendal *et al.* 1965) but only for cortical and for subcortical white matter flow measurements. The technique described here can be adapted for use in any area of the brain.

While the response of cerebral blood flow to changes in arterial P_{CO_2} are well documented, and while the ability of the brain to maintain a constant blood flow despite changes in perfusion pressure has been frequently demonstrated, nearly all reported studies of this type refer to total cerebral flow or cerebral cortical flow. Subcortical structures have received little attention; this paper records the results of an investigation designed to determine whether or not CO_2-responsiveness and autoregulation could be demonstrated in the hypothalamus of the conscious rabbit, and to compare the effects of barbiturate anaesthesia on local blood flow in the cerebral cortex and the hypothalamus.

This technique together with some preliminary results have been described elsewhere in abstract (Rosendorff & Cranston, 1969*a*, *b*).

METHODS

All experiments were performed on adult male Chinchilla rabbits, weighing 2·5–3·0 kg, in a temperature-controlled room at 22·0 ± 0·5° C. The animals were restrained in conventional stocks with their snouts gently tied into rubber rings, which were securely fixed to the stocks. In this position the rabbit was unable to move its head, but had a free range of movement of trunk and limbs. This was the simplest way to maintain a constant distance between the head and the scintillation counter. The rabbits did not appear to be at all distressed by the procedure. Before the experiments were carried out, each animal had been accustomed to this restraint by several exposures, and had shown no significant temperature change over 4–6 hr.

At least 1 week before the first experiment on each rabbit, a guide plate was attached to the skull under general anaesthesia with pentobarbitone, 30 mg/kg, intravenously. The technique has been described elsewhere (Monnier & Gangloff, 1961; Cooper, Cranston & Honour, 1965; Cranston & Rosendorff, 1967).

Hypothalamic temperature was measured in the anterior hypothalamus, using a fine bead thermistor (STC U 23 US) mounted on the end of a needle (o.d. 0·5 mm). Rectal temperature was measured with a larger thermistor (STC type G) mounted in a polyethylene tube and introduced 5–10 cm into the rectum. The thermistors were connected to bridge circuits, whose outputs were recorded continuously on a 12-channel ultra-violet recorder (SE 2005) and a digital data logging system (Solartron LY 1470). Thermistors were calibrated after each experiment.

Hypothalamic blood flow (*HBF*). HBF was measured at 15–30 min intervals by the clearance of small volumes of a saline solution of ^{133}Xe injected directly into the anterior hypothalamus (Fig. 1).

The injection system consisted of a fine (0·46 mm o.d., 0·22 mm i.d.) stainless-steel cannula, connected by a 1·5 m length of 00 nylon tubing to a 250 μl. (Hamilton) microsyringe in a micrometer syringe drive ('Agla', Burroughs Wellcome Co. Ltd).

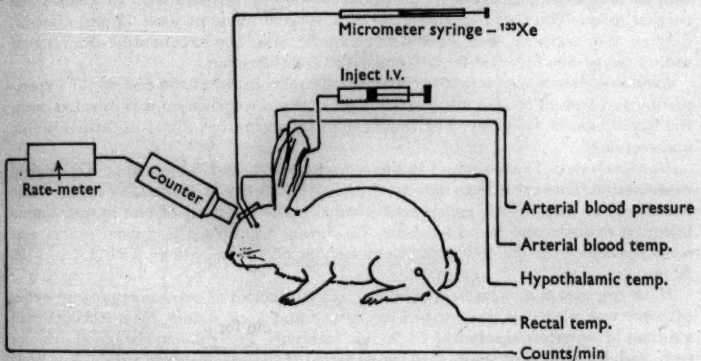

Fig. 1. Schematic diagram of the injection and recording system for ^{133}Xe.

This was filled with the ^{133}Xe-in-saline solution (5 mCi/ml.); great care was taken throughout to avoid exposure of the solution to air. The injection cannula was fixed with its tip in the anterior hypothalamus.

The recording system was a scintillation detector with a 1¾ in. diameter sodium iodide crystal (Ecko, N 609), a 3 mm thick adjustable brass collimator, and an amplifier, pulse height analyser and linear rate-meter assembly. The rate-meter output was recorded on the ultra-violet recorder and the digital data logging system.

At least 5 min was allowed for stabilization of the background, and then 2·5–10 μl. of the ^{133}Xe-in-saline solution was injected through the cannula into the hypothalamus over a 1–3 sec period. The clearance curves were then followed for at least 5 min. Injections were repeated at intervals of 15–30 min. Each curve was corrected by the subtraction of mean background activity, and re-plotted on semilogarithmic paper. The blood flows were calculated from the slope of the semilogarithmic plot of the clearance, using the formula:

$$\text{HBF (ml./100 g tissue.min)} = \frac{100.\lambda.\log_e 2}{T_{\frac{1}{2}}},$$

where $T_{\frac{1}{2}}$ is the half-decay time of the clearance curve, and $\lambda = 0{\cdot}74$, the brain: blood partition coefficient for rabbit hypothalamus (Rosendorff & Luff, 1970).

Control flows. In eight experiments on five rabbits, changes in hypothalamic blood flow were followed for 90–120 min. After two measurements of basal flow, 1 ml. normal saline was injected I.V. through a 00 nylon cannula previously inserted into a marginal ear vein. Blood flow was then followed for 90–120 min at intervals of 15–20 min. The mean basal flow for each experiment was calculated, and all subsequent flows in that experiment were expressed as a percentage of that basal value.

The no-flow situation. In one experiment a large bore polyethylene catheter was inserted, via the femoral artery, with the tip in the aorta, under pentobarbitone anaesthesia. The animal was allowed to recover, and 2–3 hr later hypothalamic Xe clearance measured. The animal was then killed by rapid exsanguination, and the clearance of Xe was again assessed.

Effect of barbiturate anaesthesia. In two experiments cortical and hypothalamic flows were measured by local injection before and after the intravenous administration of anaesthetizing doses of pentobarbitone sodium sufficient to abolish the corneal reflex. The first measurements of flow were made at least 12 min after induction of anaesthesia, and, as in other experiments, the exponential clearance of radioactivity was followed for 5–8 min after each injection.

Location of the site of injection. Five rabbits were killed at the end of the experiments, and 5 μl. of carbon ink (particulate carbon in suspension) was injected down the hypothalamic cannulae. The brains were then removed, fixed in formol saline, and sectioned.

Histopathology. Twelve rabbit brains were removed, and subjected to histological examination. These were from rabbits which had had between one and five experiments performed on them, each experiment involving the insertion of one hypothalamic injection cannula and one thermistor. The brains were fixed in formol saline, and some sections were stained with haematoxylin and eosin, others with Luxol Fast Blue.

HBF response to changes in arterial P_{CO_2}. In this series of experiments, a 00 nylon catheter was placed in the central ear artery and kept patent by injecting small volumes of heparinized saline at 20–30 min intervals. Two ml. arterial blood samples were withdrawn anaerobically via this catheter; P_{CO_2} was measured with a Severinghaus type P_{CO_2} electrode, and corrected to the animal's rectal temperature using the data of Bradley, Stupfel & Severinghaus (1956).

In four experiments on conscious rabbits, after steady rectal and hypothalamic temperature, HBF and arterial P_{CO_2} values had been achieved, a 4% CO_2-in-air mixture was administered via a looose-fitting funnel previously placed over the animal's snout. The CO_2-air mixture was continued for 6–10 min while temperature, HBF and P_{CO_2} were measured. At least two measurements of HBF and P_{CO_2} were made during the recovery phase.

Local HBF autoregulation. Forty-eight measurements of HBF were made, in seven rabbits. The rabbits were anaesthetized with pentobarbitone 30 mg/kg, supplemented when necessary during the experiment by further doses. The level of anaesthesia maintained was such that the corneal reflexes were intact. A tracheostomy was performed and the animals were respired by intermittent positive pressure ventilation, using a small animal respirator. Catheters were placed in a marginal ear vein, for intravenous anaesthesia, in a femoral vein for intravenous angiotension infusions, and in both femoral arteries for blood pressure recording and for the withdrawal of blood. HBF and rectal and hypothalamic temperatures were recorded as described above, and arterial blood pressure (B.P.) was measured using an 'Ether' blood pressure transducer. The experimental arrangement is shown in Fig. 2.

The experiment was started with a slow infusion of sterile normal saline using a Harvard infusion pump, and at least two hypothalamic blood flow measurements

were made at the starting blood pressure. Angiotensin II amide ('Hypertensin', Ciba) infusions were used to produce step-wise increments in mean arterial B.P. After at least 15 min at each new B.P. level, one or two HBF measurements were made. The 15 min stabilization period at each blood-pressure level was adopted, because of the demonstration, by Fog (1938), and Rapela & Green (1964), that following a sudden change in blood pressure there is a time lag of 30 sec to 2 min before the onset of autoregulation. The angiotensin infusion rate was then increased to produce a

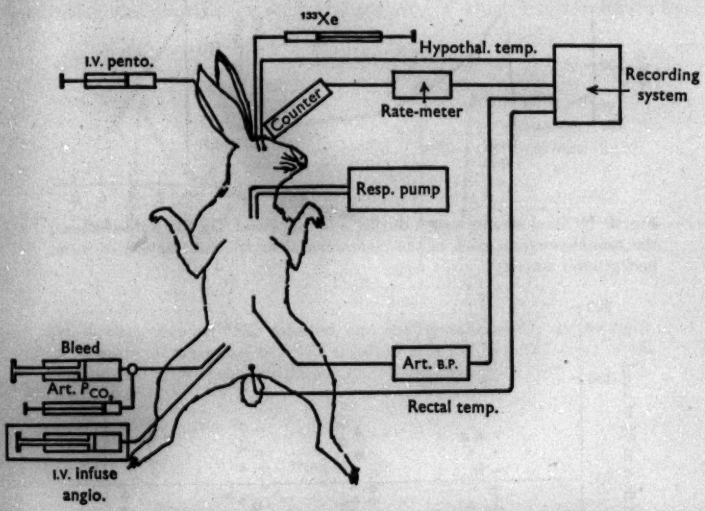

Fig. 2. Schematic representation of the experimental set-up for experiments on the autoregulation of hypothalamic blood flow.

further increase of approximately 10–20 mm Hg, and the process repeated. After blood-flow measurements at three or four increased arterial blood-pressure levels, the angiotensin infusion was discontinued, a blood-flow measurement made, and then 10–20 ml. volumes of blood withdrawn to produce step-wise decrements in mean arterial pressure. At each blood-pressure level at least one HBF measurement was made, and the arterial P_{CO_2} measured.

RESULTS

Control experiments. Clearance curves were monoexponential (Fig. 3) and the half-decay times varied from 1·00 to 2·42 min in control animals, giving values for HBF within the range 22–54 ml./100 g.min. The changes in blood flow over a 90–120 min period are shown in Fig. 4; these have been grouped in 30 min intervals. There is no significant difference between the mean of the 'basal values' (= 100 %) and the weighted means

of the 0–30, 30–60, and 60–90 min groups. The 90–120 min mean is 119% (S.E. ± 9·2%) of the basal period, and this is significantly different ($t = 2{\cdot}4$, $P < 0{\cdot}05$) from the basal flow.

The non-flow situation. Fig. 5 shows the abolition of ^{133}Xe clearance after cessation of hypothalamic perfusion.

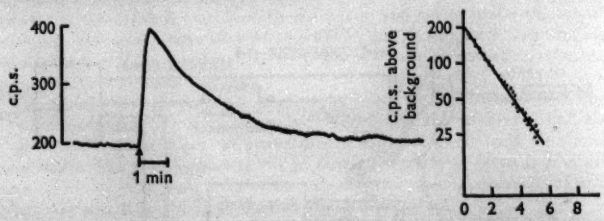

Fig. 3. ^{133}Xe clearance curve, during a basal period. On the right is shown the semilogarithmic plot of the clearance, after the subtraction of mean background activity.

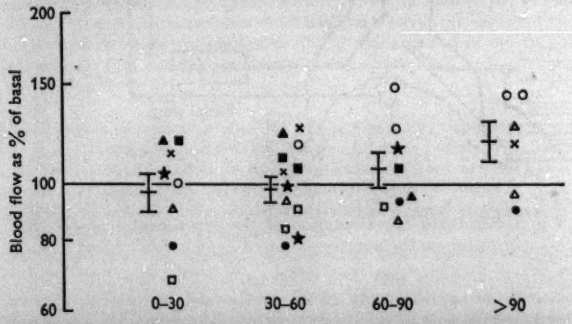

Fig. 4. Change of estimated HBF with time. Abscissa: HBF expressed as a percentage of the average of two measurements at the beginning of the experiment. Subsequent measurements grouped at 30 min intervals. An intravenous injection of normal saline was given at time 0. ■, △; □, ×; ●, ○; ▲, ★; represent different experiments on the same animal.

Effect of barbiturate anaesthesia: Fig. 6 shows a typical result. Anaesthesia with sufficient pentobarbitone to abolish the corneal reflex caused a fall in cortical blood flow from 60–70 ml./100 g.min to 23–38 ml./100 g.min. Hypothalamic flow (25–35 ml./100 g.min) was unaffected (Table 1). There was no significant departure from linearity in the semilogarithmic plots of decay of radioactivity. The effects of anaesthesia on cortical and hypothalamic blood flow differ significantly (V.R. = 179; $P < 0{\cdot}001$).

Location of the site of injection. This is shown in Fig. 7. All of the sites of injections are within 1 mm of each other and within 1 mm of the desired stereotaxic co-ordinate (aB–16 mm) in the Monnier–Gangloff system.

Histopathology. No gross disruption of hypothalamic tissue was seen in those animals which had been used in up to four experiments. In the brains of those rabbits used five or more times, oedema, inflammatory cell infiltration and gliosis were seen, the severity of which increased with the

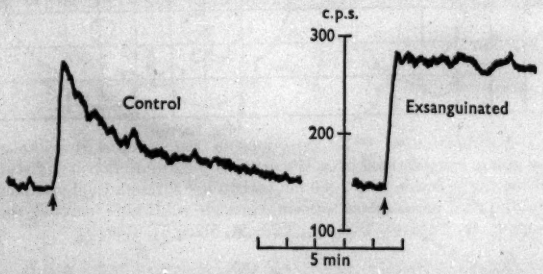

Fig. 5. Clearance of ^{133}Xe injected into the hypothalamus; on the right, the absence of clearance of ^{133}Xe injected into the hypothalamus of the same animal after exsanguination.

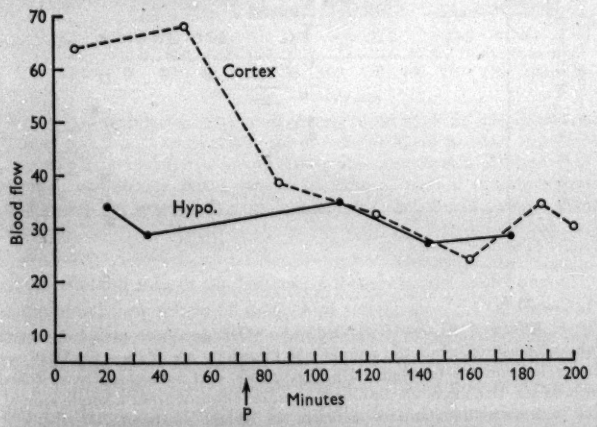

Fig. 6. Effect of pentobarbitone anaesthesia on cerebral blood flow. Ordinate: blood flow in ml./100.min. Abscissa: time (min). The open circles and dotted lines represent blood flow in the cerebral cortex (aB, 3 mm) measured by the local injection technique. The filled circles and continuous lines are hypothalamic blood flow. P is the induction of anaesthesia with 30 mg pentobarbitone/kg body wt.

number of experiments to which the animals had been subjected. Because of this finding, no animals were used for more than four experiments.

HBF response to changes in arterial P_{CO_2}. The inhalation of 4% CO_2 produced an increase of arterial P_{CO_2} of 17 mm Hg, and an increase of

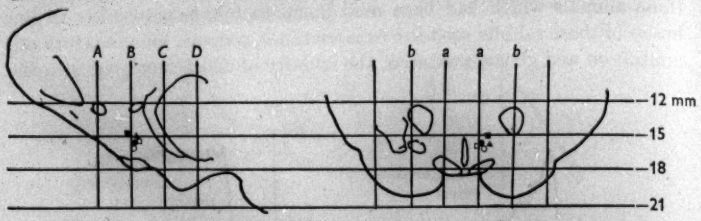

Fig. 7. Localization of ^{133}Xe injections in the anterior hypothalamus. These points were derived from the areas of maximum density of staining following the injection of 2·5–5·0 ml. carbon ink through the hypothalamic cannula. Left: parasagittal section through *a*. Right: coronal section through *B*. ●, T19; ○, T22; ▲, T28; ■, T29; □, T30.

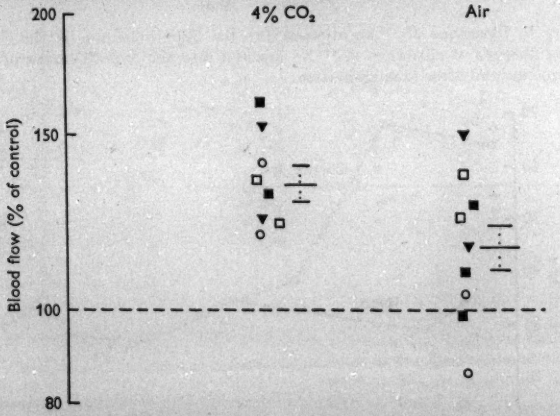

Fig. 8. Effect of CO_2 on HBF. Ordinate: HBF as a percentage of control values, measured while the animal was breathing air. Values for HBF are shown for animals breathing 4% CO_2 in air, and subsequently switched back to air. Each symbol represents results obtained from a single animal. The means and the standard errors of the means are shown.

HBF of 10 ml./100 g.min. This increase of local blood flow was associated in all cases with a transient fall of hypothalamic temperature, but with a variable rectal temperature response. The effect of the inhalation of 4% CO_2 on hypothalamic blood flow in all experiments is shown in Fig. 8.

There is a mean increase of HBF to 134% of the mean basal values, and this increase is significant (S.E. of mean $\pm$ 6·0%, $n = 8$, $t = 6{\cdot}52$, $P < 0{\cdot}0005$). After the end of the CO_2-inhalation period the mean HBF drops, but does not return to control values.

Local HBF autoregulation. Fig. 9 shows the results of a typical experiment. All blood flows except the last two lay between 30 and 40 ml./100 g.min, in spite of changes in mean arterial B.P. between 65 and 130 mm Hg. When the blood pressure fell below 30 mm Hg as a result of a

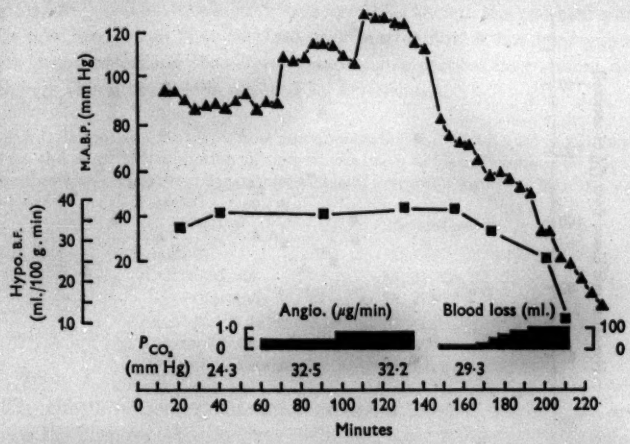

Fig. 9. Autoregulation of hypothalamic blood flow in one experiment. Traces are, from above down, mean arterial blood pressure in mm Hg, hypothalamic blood flow in ml./100 g.min, angiotensin infusion rate in μg/min, cumulative blood loss in ml. The abscissa is time in minutes, and values for arterial P_{CO_2} in mm Hg are also shown just above the abscissa.

cumulative blood loss of 80–100 ml., autoregulation failed and the hypothalamic blood flow fell to 25 and 12 ml./100 g.min. At this level of blood pressure and flow, hypothalamic temperature fell sharply. The results of all experiments of this type are shown in Fig. 10, which shows individual points obtained from each animal. The mean blood flow in a B.P. range of 81–100 mm Hg was calculated for all the initial measurements on each animal, and this was considered to be the control flow. All subsequent blood flow measurements were expressed as a percentage of the control flow. Between arterial pressures of 41 and 142 mm Hg there was no significant relationship between mean arterial pressure and blood flow (V.R. = 0·31, $P > 0{\cdot}20$). Similarly, when mean blood flows for all animals were

considered in groups for each 20 mm Hg increment of blood pressure, group mean blood flows did not deviate significantly from 100% until the blood pressure fell below 40 mm Hg (V.R. = 1·27, $P > 0·20$). The conclusion is that, at least within the range of mean arterial pressure of 41–140 mm Hg autoregulation of hypothalamic blood flow has been demonstrated. In those experiments blood pressure levels of above 140 mm Hg were not achieved, therefore no evidence has been obtained about autoregulation at mean arterial blood pressure levels above 140 mm Hg.

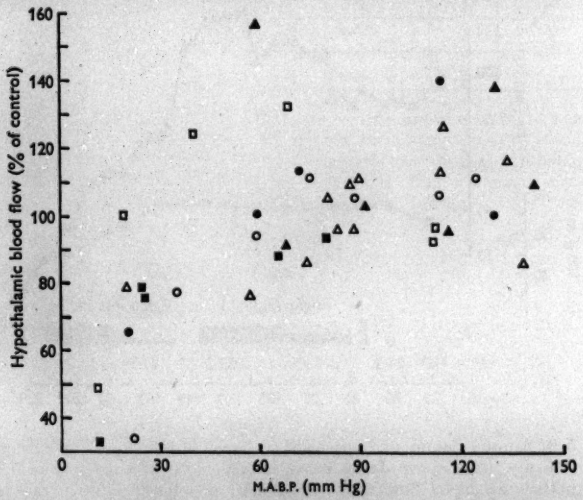

Fig. 10. Autoregulation of hypothalamic blood flow. Each value is expressed as a percentage of the mean of control blood flow measurements, made at arterial pressures between 81 and 100 mm Hg (M.A.B.P.). Different symbols represent results from different animals.

DISCUSSION

The rate of clearance of locally injected ^{133}Xe would appear to be a good index of local cerebral blood flow. The use of the initial slope of the clearance curve would seem to be theoretically valid, since this will give the fastest clearance rate, irrespective of diffusion, and is practically sound, because even if one could recognize a slow component, its activity would be so close to background levels that it would be very difficult to analyse on a compartmental basis. There is no indication from these experiments what the diffusion distance for the injected Xe is, but it would appear on the available evidence to be limited to a single compartment,

since only one component could be recognized in the clearance curves, However, the resolution of the method after the first few minutes of clearance is such that it is not possible to exclude absolutely a small slow component.

The clearance curves after injections into the cerebral cortex are also mono-exponential, within the limits imposed by the background counts, giving values for cortical flow which are higher than those for HBF (Table 1 and Fig. 6). The hypothalamus thus represents a region of sub-cortical grey matter in which flow is less than that in the cerebral cortex. It is now recognized that cerebral grey matter flow is not homogeneous, so it is not surprising that hypothalamic and cortical flow should differ, although this difference is reduced by anaesthesia.

TABLE 1. Hypothalamic blood flow measurements (ml./100 ml.min) in two animals before and after the induction of anaesthesia with pentobarbitone. The effects of anaesthesia on cortical and hypothalamic blood flow differ significantly (v.r. = 179; $P < 0{\cdot}001$)

	Control	Anaesthetized
Animal 1		
Cortex	64, 68	38, 31, 23, 32, 29
Hypothalamus	33, 29	34, 27, 28
Animal 2		
Cortex	70, 64	30, 36, 25, 37
Hypothalamus	25, 34	35, 27, 32

The effects of general anaesthetics are of interest. Barbiturate anaesthesia has been reported to lower (Schmidt, Kety & Pennes, 1945; Schieve, & Wilson, 1953), increase (Wechsler, Dripps & Kety, 1951), or have no effect (Fazekas & Bessman, 1953; Wilson, Odin & Schieve, 1953) on total cerebral blood flow. It has been suggested (Sokoloff, 1959) that changes in cerebral circulation induced by barbiturates are secondary to some of their other effects, such as changes in arterial P_{O_2}, P_{CO_2} and pH, as well as cerebral metabolic rate or oxygen consumption. In the present experiments, cortical flow was approximately halved, while there was no significant change in hypothalamic flow. This demonstrates a profound difference, not only in the blood flows in the control state, but also in the response to barbiturate anaesthesia, of two areas of grey matter.

When hypothalamic blood flow was measured repeatedly, there was a tendency for the measurements to rise slowly with time; the mean flows for groups of animals did not significantly exceed the initial flow measurements for 90 min, but after 90 min the average blood flow exceeded the control values. The reason for this change is not known.

Few studies of the effect of carbon dioxide on the local cerebral circulation have been reported. Thermocouple techniques, evolved in the 1930's,

demonstrated the ability of carbon dioxide to increase blood flow in some of the component structures of the brain, for example, the medulla (Schmidt & Pierson, 1934), the hypothalamus of the cat (Schmidt, 1934), and the parietal (Schmidt, 1936) and occipital cortex (Schmidt & Hendrix, 1937). They have not, however, provided information on the relative effectiveness of CO_2 in the various areas. The development of an autoradiographic technique, with [^{131}I]trifluoroiodomethane as the indicator (Kety, Landau, Freygang, Rowland & Sokoloff, 1955) permitted for the first time a quantitative and simultaneous determination of the effects of CO_2 on local blood flows in unanaesthetized cats (Hansen, Sultzer, Freygang & Sokoloff, 1957). The results indicate that blood flow in all regions of the brain is increased in response to CO_2, and that the increases are non-uniform, being considerably greater both absolutely and proportionately in grey matter than in white.

Perhaps the most important finding in this study is the demonstration of autoregulation in a small, subcortical area of the brain. Most reports have described results derived from flow in major arteries (Yoshida, Meyer, Sakamoto & Handa, 1966), in whole brain (Rapela & Green, 1964; Lassen, 1964), or in the cerebral cortex (Harper, 1966). Experiments have been described by Bozzao, Fieschi, Agnoli & Nardini (1968) in which blood flow, measured by a hydrogen polarographic technique, remained relatively constant in the caudate nucleus and subcortical white matter of the cat within the range of mean arterial B.P. of 70–140 mm Hg. This finding is in accord with the present study, and, on the basis of both sets of results, it can be concluded that autoregulation of cerebral blood flow is observed in subcortical grey and white structures in the same range as for the whole brain.

Many authors have pointed out that cerebral autoregulation is easily abolished, e.g. by trauma, vascular perfusion procedures, periods of circulatory arrest, arterial and local tissue hypoxia, hypercapnia, and deep anaesthesia. According to some authors (Skinhøj, 1966; Siesjö, Kjällquist, Ponten & Zwetnow, 1968) extracellular acidosis may also affect cerebral blood flow and therefore autoregulation. The preservation of autoregulatory ability under the conditions of the present investigation suggests that any trauma, hypoxia, hypercapnia or acidosis which may be induced by the presence of hypothalamic cannula or by the Xe-saline injections is not of functional significance, so far as the circulation is concerned.

This work was supported by the National Council for Research into Crippling Diseases. We are grateful to Dr N. A. Lassen for his encouragement and helpful comments during the evolution of this study.

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J. Physiol. (1971), **215**, *pp.* 591–612
With 10 *text-figures*
Printed in Great Britain

RECURRENT INHIBITION FROM MOTOR AXON COLLATERALS OF TRANSMISSION IN THE Ia INHIBITORY PATHWAY TO MOTONEURONES

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(*Received 20 July 1970*)

SUMMARY

1. The effects of impulses in recurrent motor axon collaterals on reflex transmission from different types of primary afferents to motoneurones were investigated in the cat by conditioning of PSPs evoked in motoneurones.

2. IPSPs evoked by volleys in large muscle spindle (Ia) afferents were effectively decreased when preceded by an antidromic stimulation of ventral roots. Some IPSPs from group II muscle afferents and low threshold cutaneous afferents were also slightly depressed, while other PSPs were unaffected.

3. The depression of the IPSPs could be evoked by antidromic volleys, which produced neither conductance changes in the motoneurones nor depolarization of Ia afferent terminals.

4. The effect on the Ia IPSPs is most likely due to post-synaptic inhibition of the Ia inhibitory interneurones, evoked through α-motor axon collaterals and Renshaw cells. The depression of some IPSPs from flexor reflex afferents is explained by a convergence of excitatory effects from these afferents on the Ia inhibitory interneurones.

5. The results indicate a selective recurrent control from motor axon collaterals of the interneurones in the reciprocal Ia inhibitory pathway to motoneurones.

INTRODUCTION

Impulses in motor axons can give rise to either inhibition or facilitation of motoneurones via recurrent motor axon collaterals and specific interneurones, namely Renshaw cells (Renshaw, 1941; Eccles, Fatt & Koketsu, 1954; Wilson, 1959). The facilitation is caused by the release of motoneurones from a tonic inhibitory bombardment and it has been postulated

that this disinhibition is due to recurrent inhibition of tonically active inhibitory interneurones (Wilson, 1959; Wilson & Burgess, 1962). Cells with the required properties have been found in the ventral horn (for references see Hultborn, Jankowska & Lindström, 1971*a*).

The experiments presented in this and the two following papers (Hultborn, Jankowska & Lindström, 1971*a*, *b*) were prompted by the idea that the interneurones inhibited during recurrent facilitation of motoneurones might be interposed in some inhibitory reflex pathways to motoneurones. The effects of antidromic volleys in motor axons have been investigated on reflex transmission from different types of primary afferents to motoneurones. It will be shown that the reciprocal inhibitory pathway from large muscle spindle (Ia) afferents is susceptible to recurrent inhibition, most likely at the interneuronal level. Preliminary reports of some of the results have been published (Hultborn, Jankowska & Lindström, 1968*a*, *b*).

METHODS

Preparation. The experiments were carried out on sixteen cats, some partly used for investigations presented in the two following papers. Ether anaesthesia was used during dissection, but discontinued about 3 hr before recording. Under anaesthesia the cats were anaemically decorticated as described by Andén, Jukes, Lundberg & Vyklický (1966). When the resulting rigidity was pronounced the experiments were continued without anaesthesia. In three cases of weak rigidity the animals were kept under light pentobarbitone sodium anaesthesia (Nembutal, Abbott, 25 mg/kg). Seven cats were in addition spinalized at low thoracic level. The results were similar in all three types of preparation except that the recurrent facilitatory potentials were reduced by Nembutal (cf. Fig. 3 and Wilson & Talbot, 1960). All cats were immobilized with gallamine triethiodide (Flaxedil, May and Baker Ltd.) and artificially respired. End tidal CO_2 was monitored on a Beckman medical gas analyser and kept within 4·5–5·5 % by adding a CO_2–O_2 mixture to the inspired air. Arterial blood pressure was recorded continuously. Blood loss was compensated with a mixture of low- and high-molecular-weight Dextran. A drop in blood pressure below 80 mm Hg was counteracted by slow intravenous infusion of noradrenaline (3 μg/min). Rectal temperature was kept within 37–39° C.

The spinal cord was exposed by laminectomy from L 4 to L 7. In fourteen cats the ventral roots L 5–S 1 were sectioned while in two cats the dorsal roots L 4–S 1 were cut instead. Several peripheral nerves in the left hind limb (cf. Abbreviations, below) were dissected for electrical stimulation. The nerves on the dorsal side and the cut roots were mounted on bipolar silver electrodes, while the ventral nerves were stimulated through buried electrodes. The skin flaps around exposed areas of the cord and the hind limb were sewn to form a pool filled with paraffin oil.

Recording and stimulation. Effects of antidromic volleys in motor axons on reflex transmission to motoneurones were analysed by comparing unconditioned and conditioned post-synaptic potentials in motoneurones or monosynaptic reflexes recorded from ventral roots. Different species of α-motoneurones in L 5–S 1 spinal segments were sampled intracellularly. The cells were classified according to their Ia receptiveness (Eccles, Eccles & Lundberg, 1957*b*; R. M. Eccles & Lundberg, 1958), except in the two experiments with intact ventral roots where they could be

identified antidromically. The micro-electrodes were routinely filled with 2 M-K-citrate solution but with 3 M-KCl solution when reversal of IPSPs was required. They had broken tips with diameters of 1·5–2·0 μ and resistance of 2·5–6 MΩ. The recorded potentials were fed in parallel via a cathode follower and an a.c. amplifier, to a modified Tektronix 502 oscillograph having two pairs of beams with independent time bases and to an averaging computer (CAT 1000). Usually twenty unconditioned and twenty conditioned responses were alternated in real time to two halves of the computer memory. The averaged responses were displayed on the oscillograph for photography. The resting membrane potential of the neurones was continuously monitored. The membrane conductance was measured by injecting small current pulses through the micro-electrodes. The device used allowed compensation of the voltage drop across the micro-electrode resistance (Eide, 1968). Dorsal root potentials (DRPs) were recorded from the most caudal rootlet in L6 and the excitability of primary afferent terminals was tested by the technique of Wall (1958). An a.c. amplifier used for DRP recording had a time constant of 1·6 sec.

The nerves were stimulated with square pulses of 100 μsec duration. The strength of stimulation is expressed in multiples of threshold for the lowest threshold fibres. Nerve volleys were recorded from the surface of the spinal cord with an electrode placed close to the dorsal root entry and an indifferent electrode in the back muscles. Differentiation between afferent fibre groups was done as described by Bradley & Eccles (1953), Eccles, Eccles & Lundberg (1957*a*, *c*) and R. M. Eccles & Lundberg (1959). The test stimulation was, as a rule, submaximal for the group of afferents investigated. If not otherwise stated, conditioning volleys in the ventral roots were evoked by single stimuli supramaximal for α-fibres and they preceded the test responses by 6–20 msec. The stimulus repetition rate was about 1/sec.

Abbreviations. The following abbreviations are used: anterior biceps and semimembranosus (ABSm), deep peroneus (without cutaneous and extensor digitorum brevis branches) (DP), gastrocnemius and soleus (G-S), posterior biceps and semitendinosus (PBSt), plantaris (Pl), quadriceps (Q), saphenus (Saph), sartorius (Sart), superficial peroneus (only cutaneous part) (SP), suralis (Sur), dorsal root (DR), ventral root (VR), post-synaptic potential (PSP), excitatory post-synaptic potential (EPSP), inhibitory post-synaptic potential (IPSP), recurrent inhibitory post-synaptic potential (RIPSP), recurrent facilitatory potential (RFP), dorsal root potential (DRP).

RESULTS

I. *Recurrent effects on the inhibitory pathway from Ia afferents to motoneurones*

(*a*) *Depression of Ia IPSPs*

Test Ia IPSPs were evoked in motoneurones by stimulation of nerves to antagonist muscles with the strength adjusted to excite only the lowest threshold group I afferents (Bradley & Eccles, 1953; Eccles *et al.* 1957*a*). Antidromic volleys in motor axons produced a marked depression of such IPSPs. The effect is exemplified in Fig. 1 with Ia IPSPs in a PBSt motoneurone. The test IPSPs (second column) were evoked at increasing strength of stimulation of the Q nerve. A preceding antidromic volley in the L5 and L6 VRs clearly decreased these IPSPs (third column). The conditioning volley evoked also a small recurrent facilitatory potential

(RFP), but the depression of the IPSPs was not due to the RFPs (cf. next section). In the graphs the amplitudes of the unconditioned and conditioned IPSPs (*G*) and the Ia component of the afferent volleys (*H*) have been plotted against the testing stimulation strength. The IPSP increased with the Ia component and reached its maximum below threshold for the Ib

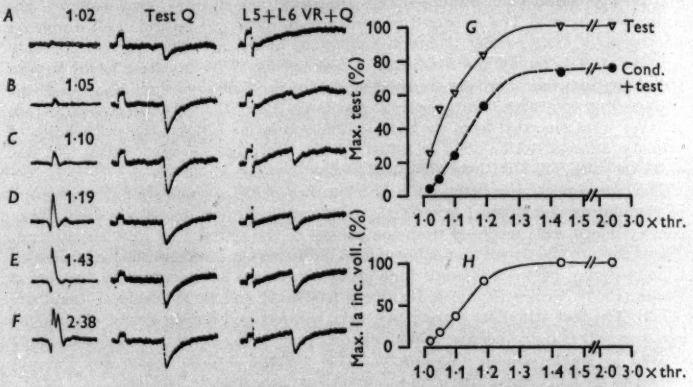

Fig. 1. Depression of Ia IPSPs in a motoneurone by antidromic volleys in motor axons. Records in the left column show afferent volleys recorded at the dorsal root entry zone. Middle and right columns show averaged intracellular records (cf. Methods) from a PBSt motoneurone. The test Ia IPSPs in the middle column were evoked from the Q nerve with the strength of stimulation (in multiples of threshold for Ia fibres) increasing from *A* to *F* as indicated above corresponding afferent volleys in the left column. The right column shows the test IPSPs conditioned by a preceding stimulation of L5 and L6 VRs. In the graph *G* are plotted the amplitudes of the unconditioned (▽) and the conditioned (●) IPSPs expressed as a percentage of the maximal unconditioned test IPSP (ordinate) against the strength of stimulation (abscissa). The amplitudes of the Ia component of the afferent volleys, expressed as a percentage of the maximal Ia component, are similarly plotted in graph *H*. Calibration pulses in each intracellular record are 0·5 mV and have 2 msec duration. Note lower amplification of intracellular records in D–F. In this and the following Figures negativity in cord surface records is signalled upwards and hyperpolarization in intracellular records downwards.

component (cf. incoming volleys in *E* and *F*). This maximal Ia IPSP was decreased to about 75% while, as generally found, the depression of the submaximal IPSPs was even more pronounced; in many cells such IPSPs were virtually abolished.

The effects of antidromic volleys in motor axons on Ia IPSPs were tested in more than 140 motoneurones innervating flexor and extensor muscles at the hip, knee and ankle joints (48 PBSt, 7 Sart, 14 DP, 46 Q,

9 ABSm, 22 G-S and 2 Pl motoneurones). Several other impaled motoneurones, particularly those of ABSm and G-S, could not be used either because the Ia IPSPs were too small to be tested or because all stimulated ventral roots evoked RIPSPs (cf. section *b*).

A clear decrease of the Ia IPSPs was found in all the tested motoneurones except six out of twenty-two G-S cells. Examples of records from different species of motoneurone are shown in Fig. 2. In each pair the upper

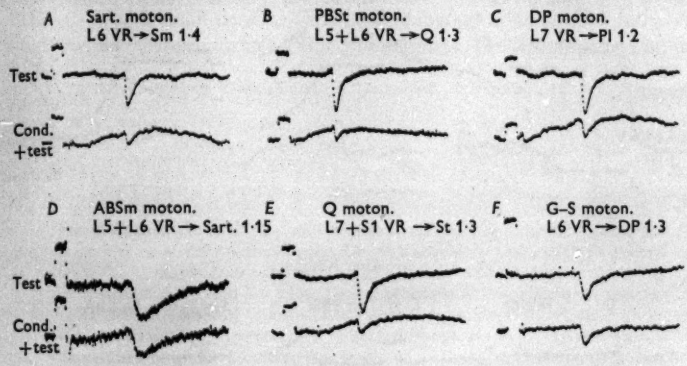

Fig. 2. Depression of Ia IPSPs in different species of motoneurone. Averaged intracellular records: *A–C* from motoneurones innervating the flexor muscles Sart, PBSt and DP; *D–F* from motoneurones supplying the extensors ABSm, Q and G-S. Upper traces in each pair show test Ia IPSPs evoked from nerves of antagonist muscles, lower traces the test IPSPs conditioned by a preceding stimulation of ventral roots. Nerves and ventral roots stimulated are indicated above the records. Duration and amplitude of calibration pulses: 4 msec and 1 mV in *A*, *C*, *E* and *F*; 4 msec and 0·5 mV in *B*; 2 msec and 0·5 mV in *D*.

trace is the unconditioned and the lower trace the conditioned response. *A–C* are from motoneurones to flexors, Sart, PBSt and DP, and *D–F* from motoneurones to extensors, ABSm, Q and G-S respectively. On the average, IPSPs evoked in motoneurones to Q, Sart, PBSt and DP by maximal Ia volleys were decreased to about 70%. Smaller effects were found in G-S and ABSm motoneurones but the explanation may be that the most effective ventral roots could not be used for conditioning stimulation since large RIPSPs were evoked from them.

Besides the decrease in amplitude, a clear increase was found in central latency of the Ia IPSPs conditioned by stimulation of the ventral roots. In four cells analysed more extensively the average increase was about 0·2 msec. There was also a greater variability in central latency of the test

responses to consecutive shocks and a small increase in time to peak of the conditioned test IPSPs.

(*b*) *Depression of Ia IPSPs in relation to permeability changes in the motoneuronal membrane*

Before the depression of Ia IPSPs could be ascribed to interference with the reflex transmission to motoneurones conductance changes in the

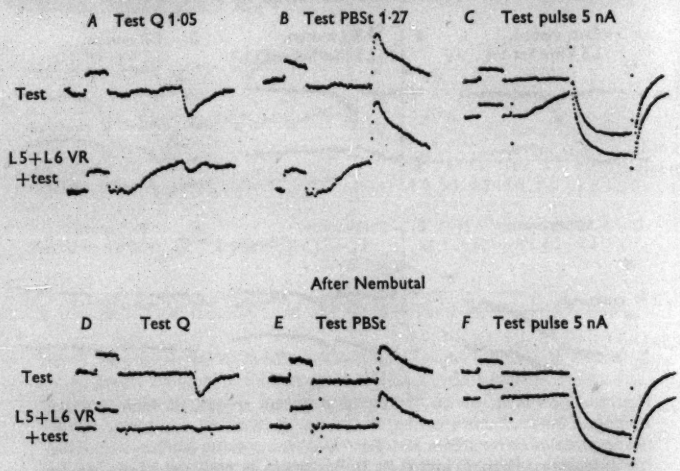

Fig. 3. Effects of Nembutal on RFPs and on the depression of Ia IPSPs. Averaged intracellular records from a PBSt motoneurone. Upper traces in each pair show the test responses: in *A* and *D* Ia IPSPs from the Q nerve, in *B* and *E* homonymous Ia EPSPs, in *C* and *F* conductance measurement pulses (5 nA). Lower traces in each pair show these test responses conditioned by a preceding stimulation of L5 and L6 VRs. Nembutal (9 mg/kg) was given intravenously a few minutes before the records in *D–F* were taken. Note that the amplitudes of the synaptic responses before and after Nembutal are not comparable because of the change in threshold of the responses caused by the anaesthetic. Calibration pulses: 0·5 mV and 4 msec.

motoneuronal membrane had to be excluded. The hyperpolarization and the increased conductance during an RIPSP can in itself reduce the amplitude of IPSPs. Thus when the antidromic volley evoked an RIPSP it was usually impossible to judge if the decrease of the test IPSP was due only to the RIPSP or to interference with the transmission in the Ia inhibitory pathway as well. However, in many motor nuclei the RIPSPs and the depression of Ia IPSPs were evoked from different ventral roots (see section (*c*) below). It was thus possible to obtain a depression of Ia IPSPs by ventral root volleys which either had no effect on the motoneurones them-

selves or evoked RFPs. The latter should not cause any decrease of Ia IPSPs but rather an increase, since the RFP is due to a release of the motoneurone from a tonic inhibitory bombardment and is associated with a decreased conductance of the motoneuronal membrane (Wilson & Burgess, 1962).

As the recurrent facilitation is very sensitive to barbiturate anaesthesia (Wilson & Talbot, 1960) an injection of Nembutal was used to show that the depression of Ia IPSPs can be dissociated from RFPs. Fig. 3 illustrates the effect of a small dose of Nembutal (9 mg/kg) administered intravenously while recording from a PBSt motoneurone. The test responses (upper

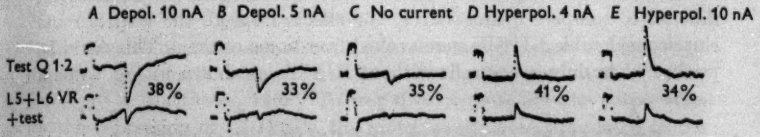

Fig. 4. Depression of normal and reversed Ia IPSPs. Averaged intracellular records from a PBSt motoneurone. Upper traces show test Ia IPSPs evoked from the Q nerve, lower traces the test IPSPs conditioned by a preceding stimulation of L5 and L6 VRs. The amplitude and the polarity of the test IPSP was successively changed from *A* to *E* by current injection through the recording KCl electrode, as indicated above records. The percentage depression is given between the traces in each pair. Calibration pulses: 0·5 mV and 4 msec.

traces) were the Ia IPSP (*A* and *D*), the homonymous Ia EPSP (*B* and *E*) and a small hyperpolarizing current pulse injected through the microelectrode (*C* and *F*). These tests were conditioned by a preceding antidromic volley in the L5 and L6 VRs (lower traces). Without anaesthesia the antidromic volley evoked a large RFP (*A–C*) which was completely abolished by Nembutal (*D–F*). During the RFP there was a conductance decrease of about 10% as compared with the resting conductance (*C*) which can fully explain the increase in amplitude of the conditioned Ia EPSP in *B*. When the RFP was abolished there was no change in amplitude of the Ia EPSP (*E*) or the test pulse (*F*), indicating that the conditioning volley did not evoke any conductance change in the motoneuronal membrane, detectable from the soma. The Ia IPSP was, however, still effectively depressed (*D*).

In motoneurones which were progressively depolarized the ratio between the unconditioned and the conditioned IPSPs was the same at different membrane potential levels. This was the case also when the Ia IPSPs were reversed in depolarizing direction as illustrated in Fig. 4. The upper records show Ia IPSPs evoked in a PBSt motoneurone by stimulation of the Q nerve with the same strength throughout. A conditioning anti-

dromic volley in the L5 and L6 VRs markedly decreased these IPSPs (lower records). Passage of depolarizing and hyperpolarizing current through the KCl-filled micro-electrode changed the amplitude and the polarity of the Ia IPSP. The ratio between the unconditioned and the conditioned IPSPs remained constant, however, throughout the series. Note that the small RFP seen in *A–C* is clearly reversed in hyperpolarizing direction in *E*.

(*c*) *Effective ventral roots*

Volleys in different ventral roots were not equally effective in depressing Ia IPSPs in a given species of motoneurone. In most cells it was, however, difficult to compare quantitatively the effects from all the ventral roots since considerable RIPSPs were evoked from some of them. This could be performed in only a few cells without RIPSPs as shown in Fig. 5 for a

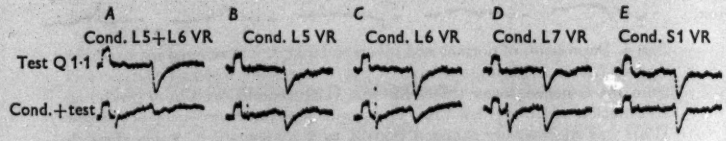

Fig. 5. Comparison of Ia IPSP depression evoked from different ventral roots. Averaged intracellular records from a PBSt motoneurone located in caudal L7. Upper traces show test Ia IPSPs evoked from the Q nerve, lower traces the test IPSPs conditioned by stimulation of different ventral roots as indicated above each set of records. The strength of the ventral root stimulation was supramaximal for α-fibres, except in *D* where it was just subthreshold for antidromic invasion of the motoneurone investigated. Note that the stimulation in *D* was strong enough to activate several other motoneurones as indicated by the field potential close to the calibration pulse in the lower trace. Calibration pulses: 0·5 mV and 2 msec.

PBSt motoneurone located in the caudal part of the L7 segment. The test Ia IPSPs were evoked by a submaximal group Ia stimulation of the Q nerve. In the upper row are the unconditioned test responses while the corresponding records in the lower row show the effect of antidromic volleys in different ventral roots. The conditioning stimulation was supramaximal for α-fibres, except in the case of the L7 VR which was stimulated just subthreshold for antidromic invasion of the cell. The latter stimulation was, however, strong enough to fire several neighbouring motoneurones as indicated by the antidromic field potential (lower trace in *D*). Fig. 5 illustrates the typical finding that the Ia IPSPs in PBSt motoneurones were depressed by volleys in the L5 and L6 VRs while volleys in the L7 and S1 VRs were without significant effect. In Q motoneurones, on the other hand, the Ia IPSPs from the PBSt nerve were

reduced from the L7 and S1 VRs. Observe also in Fig. 5 that combined stimulation of the L5 and L6 VRs was more effective (record *A*) than separate stimulation of either of them (records *B* and *C*).

In unanaesthetized animals RFPs were evoked in Q and PBSt motoneurones by volleys in the same ventral roots which depressed the Ia IPSPs. The recurrent inhibition was mainly evoked from the other roots (L7 and S1 VRs in PBSt motoneurones and L5 and L6 VRs in Q motoneurones). Different segmental origin of the RFPs and the effects on Ia IPSPs, on one hand, and of the RIPSPs on the other, was seen also in the other species of motoneurones, though less clearly in motoneurones to ABSm and G-S.

(d) Contribution from α- and γ-efferents to the depression of Ia IPSPs

The recurrent inhibition of both α- and γ-motoneurones is caused by impulses in α-motor axons (Renshaw, 1941; Ellaway, 1968; Brown, Lawrence & Matthews, 1968). To test if α-efferents are also responsible for the depression of Ia IPSPs, the motor axons were stimulated in the peripheral nerves. It was then possible to control the threshold separation between α- and γ-fibres by recording from a cut ventral rootlet (cf. Leksell, 1945). These experiments were performed on preparations with transected dorsal roots in which the test Ia IPSPs were evoked by very weak stimulation of dorsal roots containing Ia afferents from antagonists (cf. Hultborn *et al.* 1971*b*).

An example of a disynaptic IPSP evoked in a PBSt motoneurone by very weak stimulation of the L5 DR is shown in Fig. 6*B*. Records *C–F* and graph *A* illustrate the effect on this IPSP of conditioning antidromic stimulation of the Q nerve with increasing strengths. Graph *G* gives the relation between the strength of stimulation and the size of the volleys in α- and γ-fibres, recorded from a cut rootlet of the L5 VR. The two graphs show that the conditioned Ia IPSP decreased approximately in parallel with the increase of the α-volley and reached a plateau at a stimulus strength just maximal for α-fibres. Similar results were obtained from six other motoneurones of different types.

The finding that no additional effect was produced when the stimulus strength was increased to activate γ-fibres does not exclude that γ-efferents might contribute to the recurrent effects. If they have collaterals which converge on the same Renshaw cells as the α-motor-axon collaterals the plateau of the depression could be due to occlusion. However, this explanation is very unlikely in view of the marked parallelism between the depression of the Ia IPSPs and the increase of the α-volleys.

(e) Time course of the recurrent depression of Ia IPSPs

Recurrent inhibition and facilitation of motoneurones by single antidromic volleys have a typical prolonged time course which has been attributed to the repetitive firing of Renshaw cells (Eccles *et al.* 1954).

The central latencies of the effects indicate a disynaptic pathway for the recurrent inhibition (Eccles *et al.* 1954) and a trisynaptic path for the recurrent facilitation (Wilson, 1959; H. Hultborn, E. Jankowska &

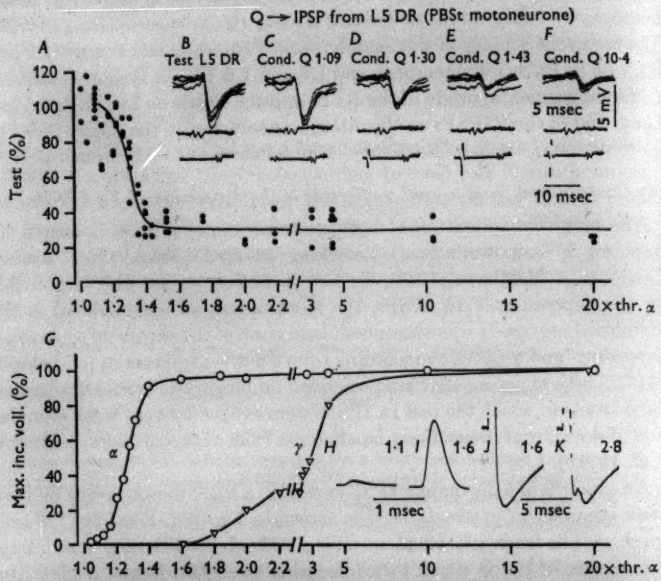

Fig. 6. Effects from α- and γ-efferents. Graph *A* and sample records in *B–F* show the depression of Ia IPSPs in a PBSt motoneurone by conditioning stimulation of efferent fibres with increasing strength. Upper traces in *B–F* show intracellular potentials. Middle and lower traces are from the dorsal-root entry zone; the lower recorded with a slower sweep speed. The test Ia IPSP (*B*) was evoked by very weak stimulation of the L 5 DR. The conditioning stimulation was applied to the Q nerve (*C–F*; conditioning–testing interval constant). The stimulus strength is expressed in multiples of threshold for α-fibres. In the graph *A* are plotted the amplitudes of the conditioned IPSPs expressed as a percentage of the unconditioned test IPSPs (ordinate) against the strength of conditioning stimulation (abscissa). Each point represents the average amplitude of five responses. Graph *G* shows the relation between the strength of conditioning stimulation (abscissa) and the size of the antidromic volleys in α- (○) and γ- (△) efferents expressed as a percentage of the maximal α- and γ-volleys respectively (ordinate). The volleys were recorded from a dissected rootlet of the L 5 VR shortly after recording the series in *A*. The inserted records show the α-volleys (*H* and *I*) and the γ-volleys (*J* and *K*) at indicated strengths of stimulation. Note higher amplification and slower sweep speed in *J–K*.

S. Lindström, unpublished). The latency and time course of the recurrent depression of Ia IPSPs in motoneurones are shown in the graphs in Fig. 7. A disynaptic test IPSP was evoked in a Sart motoneurone by very weak stimulation of the L6 DR (*C*). The conditioning stimulation, supramaximal for α-fibres, was applied to the Sm nerve (*D–F*). The conditioning volley had to precede the test volley by at least 0·6 msec to evoke the depression. The effect was maximal at an interval of about 8 msec and

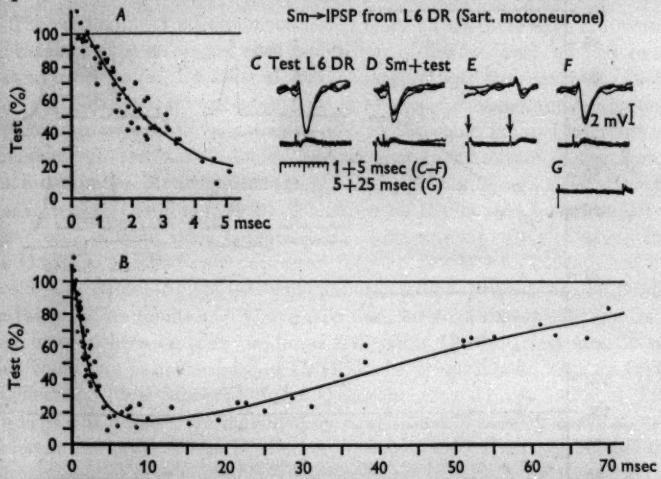

Fig. 7. Time course of the recurrent depression of Ia IPSPs. Upper traces in records *C–F* show intracellular potentials in a Sart motoneurone. Lower traces are from the dorsal root entry zone in L5. Record *C* shows a test Ia IPSP evoked by weak stimulation of the L6 DR. *D–F* show the test IPSP conditioned at different time intervals by an antidromic volley in the Sm nerve (the conditioning–testing interval of *F* is shown in *G*, recorded with a slower sweep speed). In graphs *A* and *B* the amplitudes of the conditioned IPSPs expressed as a percentage of the unconditioned test IPSPs (ordinates) are plotted against the time interval between the conditioning and the testing volleys (abscissae). The time intervals were measured as indicated by arrows in *E*. Each point represents the average amplitude of five responses.

lasted for some 80 msec. Similar results were obtained with the test Ia IPSPs evoked by stimulation of peripheral nerves and conditioning stimulation of ventral roots. In sixteen motoneurones the minimal effective interval between the antidromic and the Ia volleys ranged between 0·3 and 0·9 msec (average 0·5 msec). The time to peak of the depression varied between 5 and 12 msec (average 8 msec) and the duration between 50 and 100 msec (average 75 msec).

The depression of the Ia inhibition of motoneurones could be demonstrated also when tested on the Ia inhibition of monosynaptic reflexes although care had to be taken that the results were not obscured by recurrent facilitation. The time course of the recurrent effects found in this way is illustrated in Fig. 8. The records in *C* show the unconditioned PBSt

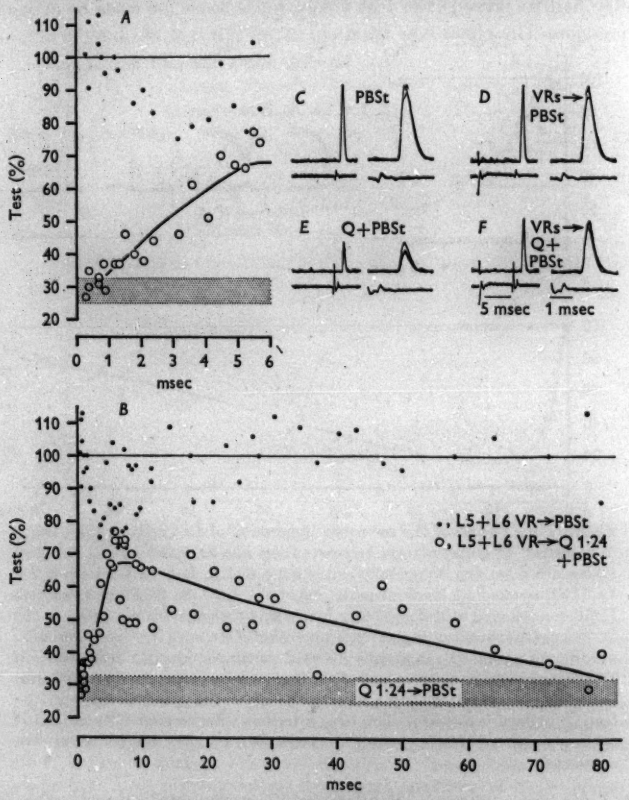

Fig. 8. For legend see opposite page.

test reflex at two different sweep speeds. In *E* and *F* this test is conditioned by a Ia volley in the nerve to the antagonist Q. In *F* the inhibition is much weaker because of a preceding antidromic volley in the L5 and L6 VRs. The latter stimulation alone did not modify the test reflex in a significant way (*D*). Records *C*–*F* are samples from the series plotted in the graphs.

The antidromic stimulation alone seemed to produce some early recurrent inhibition of the PBSt motoneurones but no recurrent facilitation. The increase of the Ia inhibited test by the antidromic volley therefore demonstrates the decrease of the Ia inhibitory action.

II. *Recurrent effects on other reflex pathways to motoneurones*

(*a*) *Effects on Ib IPSPs*

The predominant effects encountered from Ib afferents were inhibition of extensor motoneurones and facilitation of flexors, both evoked from extensor nerves (cf. Eccles *et al.* 1957*c*). Accordingly the recurrent effects on Ib IPSPs were investigated only in extensor motoneurones. The test IPSPs were evoked by stimulation of high-threshold group I afferents in different extensor nerves known not to give Ia inhibition of the motoneurones under investigation (R. M. Eccles & Lundberg, 1958; Hongo, Jankowska & Lundberg, 1969). Effects on Ib IPSPs were tested in forty-three cells, most of them Q motoneurones (twenty-six cells), in which the Ia IPSPs were effectively depressed. The Ib IPSPs were conditioned by antidromic stimulation of the ventral roots used to condition the Ia IPSPs in the cells. As mentioned above, the other ventral roots could not as a rule be used because they produced RIPSPs in the motoneurones. In no case could any clear decrease of Ib IPSPs be demonstrated. One example of effects on Ib IPSPs evoked in a Q motoneurone is given in Fig. 9. The Ib IPSPs in *B* and *C* were evoked by stimulation of the G-S nerve at two different strengths. These IPSPs were not decreased when conditioned by

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Legend to Fig. 8.

Fig. 8. Effects on the Ia inhibition of monosynaptic reflexes. Upper traces in records *C–F* show monosynaptic reflexes recorded in L7 VR, lower traces are from the L6 dorsal root entry zone. The same responses are shown with two different sweep speeds (cf. calibrations in *F*). The test reflex (*C*) was evoked by stimulation of the PBSt nerve. This test was inhibited by a preceding volley in Ia afferents from Q (*E*). Following a stimulation of L5 and L6 VRs the Ia inhibition of the test reflex was less pronounced (*F*). The antidromic volley alone had no influence on the test reflex (*D*). In the graphs *A* and *B* the amplitudes of monosynaptic reflexes conditioned by stimulation of ventral roots (ordinates) are plotted against conditioning–testing intervals (abscissae). The amplitudes of the conditioned responses are expressed as a percentage of the unconditioned test reflex. Dotted area shows the mean amplitude (with a 95 % confidence interval) of the Ia inhibited test reflex (as in *E*). ●, Test reflex conditioned by ventral root stimulation (as in *D*); ○, the Ia inhibited test reflex conditioned by ventral root stimulation (as in *F*). Time intervals were measured between the arrival at the spinal cord of the ventral root volley and the PBSt volley (●) or the Q volley (○). Each point represents the average amplitude of three test reflexes.

volleys in the L7 and S1 VRs but slightly increased due to the small RFP. As shown in *A*, antidromic stimulation of the same ventral roots effectively decreased the Ia IPSP from the St nerve.

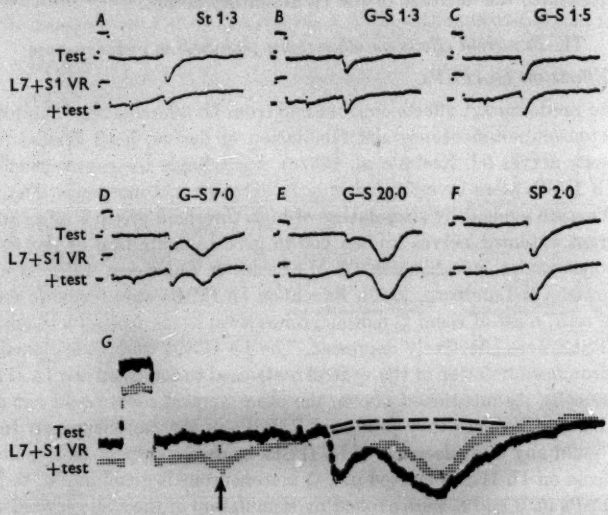

Fig. 9. Effects on IPSPs from different groups of afferents. Averaged intracellular records from a Q motoneurone. Upper traces in records *A–F* show test IPSPs evoked from different groups of afferents: *A*, group Ia afferents in the St nerve; *B* and *C*, group Ib afferents in the G-S nerve, stimulated with two different strengths; *D*, group Ib and II afferents in the G-S nerve; *E*, the latter plus group III afferents; *F*, low-threshold cutaneous afferents in the SP nerve. Lower traces show these IPSPs conditioned by an antidromic volley in L7 and S1 VRs. *G* shows the two traces of record *D* superimposed. The response to ventral root stimulation alone has been added as a double interrupted line. Further details in the text. Calibration pulses: 1 mV and 4 msec.

(*b*) *Effects on FRA IPSPs*

From the point of view of their reflex actions group II and III muscle afferents, cutaneous afferents and high-threshold joint afferents are often grouped together as flexor reflex afferents (FRA; R. M. Eccles & Lundberg, 1959). In the acute spinal cat their principal effects to motoneurones are excitation of flexors and inhibition of extensors. Under certain conditions the FRA may instead evoke IPSPs in flexor motoneurones and EPSPs in extensor motoneurones, indicating the existence of alternative pathways from the FRA to motoneurones (R. M. Eccles & Lundberg,

1959; Holmqvist & Lundberg, 1961). Recurrent effects on FRA IPSPs were investigated mainly in extensor motoneurones, but also in some flexor motoneurones (total fifty-seven motoneurones). The IPSPs were evoked from different muscle and cutaneous nerves and from the posterior nerve to the knee joint of the ipsilateral hind limb. In most cells the antidromic volleys were without any effect on the FRA IPSPs (Fig. 9*F*). However, a small depression was found of some IPSPs evoked from group II muscle afferents and/or low-threshold cutaneous afferents. The depression was less pronounced than that of Ia IPSPs even if the submaximal FRA IPSPs were smaller than the Ia IPSPs. The effect was not due to a conductance change in the motoneuronal membrane.

Fig. 9*D* and *G* exemplifies the decrease of an IPSP evoked from high-threshold muscle afferents. The records are from the same Q motoneurone in which the Ib IPSPs (*B–C*) were investigated. In record *D* the stimulation of the G-S nerve was increased to activate also group II afferents. The conditioning volley did not change the early Ib component of the IPSP (cf. records *B* and *C*), but the second component evoked from group II afferents was delayed and decreased. This is best seen in *G* with superimposed traces of the unconditioned and conditioned tests. Record *E* shows the effect of the same conditioning volley when the testing stimulus was increased to activate group III muscle afferents. The early part of the FRA IPSP (evoked from group II afferents) was still delayed by the conditioning volley but there was no depression of the main component. In fact the amplitude of the conditioned FRA IPSP in *E* was even slightly larger than of the unconditioned test, presumably due to the small RFP. Depression of group II IPSPs was found in eight Q, two AB and three PBSt motoneurones of twenty-eight tested. When group II IPSPs were evoked in the same Q cells from several nerves only some of them were decreased while the others were unaffected.

Decrease of IPSPs from low-threshold cutaneous afferents was found in six Q and two PBSt motoneurones of twenty-seven tested cells. All the affected IPSPs were evoked from the Sur nerve, while IPSPs from the SP and Saph nerves were not significantly depressed. Likewise no depression was found of IPSPs from high-threshold cutaneous afferents. The IPSPs evoked from the Sur nerve were depressed in Q motoneurones from the L7 and S1 VRs but in PBSt motoneurones from the L5 and L6 VRs. This indicates that in a given motoneurone FRA IPSPs can be depressed from the same ventral roots as the Ia IPSPs.

(c) *Recurrent effects on EPSPs*

Recurrent effects on EPSPs from different afferent systems were studied less extensively than the effects on IPSPs. Only effects from ventral roots

which did not evoke RIPSPs in the motoneurones could be analysed. Fig. 3*E* illustrates that the amplitude of monosynaptic Ia EPSPs was unchanged by antidromic volleys which effectively depressed the Ia IPSPs in the same cells, provided the conditioning stimulation did not evoke RFPs (as in *B*) or RIPSPs.

The excitatory pathways from Ib afferents and the FRA include one or more interneurones which might be influenced by volleys in motor axon collaterals. No effect was found on Ib EPSPs tested in a few motoneurones.

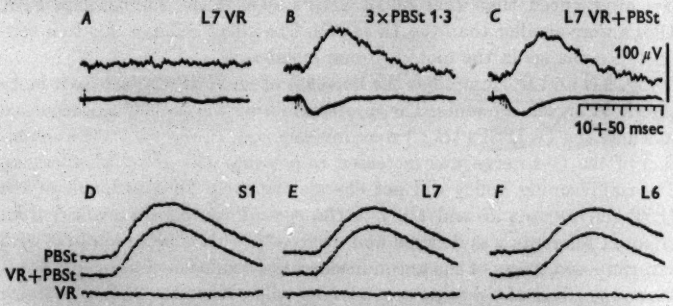

Fig. 10. Effects on DRPs evoked by Ia volleys. Upper traces in *A–C* are single sweep records from the most caudal rootlet in L6, lower traces are from the dorsal root entry zone. *A* demonstrates that no DRP was evoked by single antidromic stimulation of the L7 VR. The DRP in *B* was evoked by three Ia volleys in the nerve to the flexor PBSt. *C* shows this DRP conditioned by a preceding single stimulation of the L7 VR. *D–F* are averaged records from the same rootlet. Upper traces show DRPs evoked by PBSt volleys as in *B*. Middle traces show these DRPs conditioned by a single antidromic volley in the S1, L7 and L6 VRs respectively, and the lower traces the effects of the antidromic volleys alone.

Neither were EPSPs evoked from the FRA decreased. In a few cells a small increase in amplitude of FRA EPSPs was found, but this effect was probably due to small RFPs.

III. *Recurrent effects on primary afferent terminals*

The observed depression of group Ia IPSPs by impulses in motor axon collaterals could be due to presynaptic inhibition of the transmission from Ia primary afferents to the inhibitory interneurones. This seemed unlikely as in the cat antidromic stimulation of ventral roots neither evokes a 'P-wave' in the cord dorsum recording (Gasser & Graham, 1933) nor DRPs (Barron & Matthews, 1938). Further, Wall (1958) was unable to demonstrate any change in excitability of G-S group I afferent terminals in the ventral horn after antidromic stimulation of the S1 VR.

We have confirmed that DRPs are not evoked by antidromic stimulation of ventral roots (Fig. 10*A* and lower traces in *D–F*). The finding that ventral root volleys do not depress Ia EPSPs (Fig. 3*E*) also indicates that such volleys do not depolarize Ia terminals on motoneurones. However, selective effects on Ia terminals on the Ia inhibitory interneurones cannot be entirely excluded since the electrotonic spread of the terminal depolarization of Ia afferents might be limited by the length or small diameter of the preterminal part. The excitability of Ia afferents was therefore tested as described by Wall (1958) with the stimulating electrode placed dorsomedially to the motor nuclei in a region where Ia collaterals terminate (Szentágothai, 1967) and interneurones supposedly mediating Ia inhibition have been found (Hultborn *et al.* 1971*a*). Neither in this case could any effect of ventral root stimulation be demonstrated on Ia terminals.

The influence of volleys in motor axon collaterals on the transmission in the pathway from Ia afferents to Ia afferent terminals was also tested. This was done by conditioning DRPs evoked by a train of group Ia volleys in a flexor nerve, which are known to evoke depolarization of Ia afferent terminals (Eccles, Magni & Willis, 1962). In no case were these DRPs decreased by single shock or repetitive stimulation of the ventral roots. This is exemplified in Fig. 10 (*B*, *C* and upper and middle traces in *D–F*) with DRPs evoked by Ia volleys in the PBSt nerve. DRPs evoked by volleys in group Ib muscle afferents or low-threshold cutaneous afferents were likewise unaffected.

DISCUSSION

The present results demonstrate that impulses in recurrent motor axon collaterals, besides the well-known inhibitory effects on motoneurones, also affect transmission of synaptic actions to motoneurones. The effect is probably restricted to inhibition of the interneurone in the reciprocal Ia inhibitory pathway to motoneurones.

The effects of impulses in recurrent motor axon collaterals on reflex transmission to motoneurones were deduced from changes of PSPs in motoneurones after antidromic stimulation of ventral roots. Various evidence has been brought to establish that the observed depression of Ia IPSPs is not due to post-synaptic effects in the motoneurones. In most cells the depression could be produced from ventral roots which did not evoke any detectable RIPSPs. In unanaesthetized animals the effective antidromic volleys instead often evoked RFPs. The depression of the IPSPs, however, did not depend on these RFPs, as the effect could be evoked also when the RFPs were abolished by barbiturate. The effective

antidromic volleys did not then produce any detectable conductance change in the motoneurone when tested with small current pulses. Conductance changes in more remote parts of the dendrites, not revealed by micro-electrodes placed in the soma, are not likely to be of importance, since the synapses of the Ia inhibitory interneurones are most probably localized close to the soma of the motoneurones (Curtis & Eccles, 1959; Smith, Wuerker & Frank, 1967; Burke, Fedina & Lundberg, 1968). Monosynaptic Ia EPSPs and IPSPs from other afferent systems were also unaffected by the ventral root stimuli which effectively decreased the Ia IPSPs. The constant ratio between the unconditioned and the conditioned Ia IPSPs during injury depolarization of the motoneurones or reversal of the IPSPs by injection of chloride ions further demonstrates that the depression of the Ia IPSPs is independent of the state of the motoneurones. The remote possibility that the IPSP depression represented summation of the IPSP with an EPSP in some released disynaptic pathway from Ia afferents is excluded by the observation that the hyperpolarizing and the reversed Ia IPSPs were depressed to the same extent. If an EPSP was present the test response should have been increased on conditioning by addition of an EPSP to the reversed IPSPs. All the evidence thus leads to the conclusion that the depression of Ia IPSPs is due to inhibition of the transmission in the Ia inhibitory pathway, either presynaptically by depolarization of Ia afferent terminals, or at the interneuronal level.

Antidromic volleys in ventral roots are known to evoke primary afferent depolarization in the frog (Barron & Matthews, 1938) and it has recently been reported that such volleys may also give some discharges in primary afferents also in the cat (Decima, 1969; Decima & Goldberg, 1970). However, in cats this effect seems to be very marginal and is probably unrelated to presynaptic inhibition. It may be caused by ephaptic excitation of a few depolarized presynaptic terminals by motoneuronal action currents. Observe that neither P-waves nor DRPs are evoked by antidromic stimulation of ventral roots in the cat (Gasser & Graham, 1933; Barron & Matthews, 1938). Furthermore, conditioning ventral-root stimuli did not give any change in the excitability of Ia afferent terminals stimulated in the region where the presumed Ia inhibitory interneurones have been found. There are therefore no indications that the recurrent depression of Ia IPSPs in motoneurones is caused by depolarization of group Ia afferent terminals. It follows that the effect must be exerted on the interneurone interposed in the Ia inhibitory pathway either by post-synaptic inhibition or by presynaptic inhibition of the transmission from their terminals to motoneurones.

The duration of the recurrent depression of the Ia IPSPs is shorter than

that generally found for presynaptic inhibition and corresponds more with the time course of RIPSPs in motoneurones. The Ia IPSPs are depressed when the antidromic volleys arrive at the spinal cord only 0·3–0·9 msec in advance of the Ia volleys. This small difference in the central latency of the effects allows for only one more synapse in the recurrent inhibitory path to the interneurones than in the excitatory path from Ia afferents. Thus the recurrent inhibitory pathway to the interneurones must be disynaptic like that to the motoneurones. Most likely the recurrent depression of the Ia IPSPs in motoneurones is mediated through motor axon collaterals and Renshaw cells, which evoke post-synaptic inhibition of the Ia inhibitory interneurones. This suggestion is supported by the finding that the central latency and the time to peak of the Ia IPSPs in motoneurones are increased by conditioning volleys in motor axons, indicating an interference with the spike generation in the interneurones. Moreover, interneurones with convergence of monosynaptic Ia EPSPs and disynaptic IPSPs from motor axon collaterals have been identified and will be reported in a following paper (Hultborn *et al.* 1971*a*).

In all cases in which the motor nuclei to antagonists were located at different segmental levels the motor axons giving RIPSPs in one species of the motoneurone and those producing depression of Ia IPSPs in the same cells seemed to have different segmental distribution. The RIPSPs were mainly evoked from ventral roots of the segments in which the motor nuclei investigated were located, which confirms earlier findings that the recurrent inhibition is most pronounced between the motor nuclei within the same segment (Renshaw, 1941; Eccles *et al.* 1954). The Ia IPSPs, on the other hand, were decreased after stimulation of ventral roots from the segments where the Ia afferents, giving rise to the test IPSPs, entered the spinal cord. This finding raised the question whether the depression of the Ia IPSPs in a given species of motoneurones is mediated via axon collaterals of motoneurones supplying antagonists rather than of those innervating synergists. That this is indeed the case will be shown in a following paper (Hultborn *et al.* 1971*b*).

In all motor nuclei investigated the Ia IPSPs could be depressed by antidromic volleys in motor axons. No effect was found on PSPs from other groups of afferents tested with the exception of some IPSPs from group II muscle afferents and low-threshold cutaneous afferents. Because of the scanty effects on such IPSPs a more detailed analysis of the effects could not be performed. Using the same criteria as for the Ia IPSPs it could be shown, however, that the decrease of these FRA IPSPs was due to recurrent inhibition exerted at an interneuronal level. It is therefore of great interest that Ia IPSPs in motoneurones can be facilitated by weak FRA stimulation, due to a spatial summation on the Ia inhibitory inter-

neurones (cf. Lundberg, 1970). Furthermore, the interneurones with convergence of monosynaptic Ia excitation and disynaptic inhibition from motor axon collaterals are also excited by the FRA (Hultborn *et al.* 1971*a*). It is thus likely that volleys in the FRA may evoke inhibition in motoneurones at least partly by activation of Ia inhibitory interneurones. If so, the IPSPs evoked via this route in a given motoneurone should be susceptible to recurrent depression from the same ventral roots as the Ia IPSPs, and this was found to be the case. There is now evidence for a 'private' inhibitory pathway from the FRA to motoneurones in which the interneurones are not shared with Ia afferents (cf. Lundberg, 1970). Our results are well explained if it is assumed that the interneurones of the latter pathway do not receive recurrent inhibition from motor axon collaterals. The inconsistency of the effects on FRA IPSPs may be explained by the fact that the IPSPs to different extent are mediated by one or the other of these two pathways from the FRA. When the FRA IPSPs are not depressed they may be mediated largely by the 'private' FRA pathway while in other cases both interneuronal pathways may contribute. A coactivation of the two pathways may explain why in the same cells we found a smaller depression of submaximal group II IPSPs than of Ia IPSPs.

The present results thus suggest that *the recurrent effects on segmental reflex pathways to motoneurones are restricted to inhibition of the interneurones in the reciprocal Ia inhibitory pathway*. The high selectivity of the recurrent control of the Ia inhibitory interneurones suggests that recurrent inhibition has a very specific function in motor control and this is discussed in a following paper (Hultborn *et al.* 1971*b*).

The authors wish to express their sincere gratitude to Professor Anders Lundberg for many inspiring discussions and for his invaluable help throughout the investigation and in the preparation of the manuscript. Our thanks are also due to Dr Simon Miller for scrutinizing the English and to Mrs Rauni Larsson for excellent technical assistance. This work was supported by the Swedish Medical Research Council (Project No. B70-14X-94-06B).

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With 9 text-figures
Printed in Great Britain

RECURRENT INHIBITION OF INTERNEURONES MONOSYNAPTICALLY ACTIVATED FROM GROUP Ia AFFERENTS

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(*Received 20 July 1970*)

SUMMARY

1. Interneurones monosynaptically excited from large muscle spindle (Ia) afferents and inhibited from motor axon collaterals were searched for in the lumbar spinal cord of the cat.

2. Monosynaptic Ia excitation was found in sixty-seven of sixty-nine interneurones inhibited by antidromic volleys. These interneurones were excited from Ia afferents from one or a few muscles (mainly close synergists). Volleys in high threshold muscle and skin afferents (FRA) evoked polysynaptic excitation or inhibition. Weak inhibition from Ia afferents (from antagonists to those giving Ia excitation) was seen in a few cells. Monosynaptic excitation was evoked from the ventral quadrant of the spinal cord and polysynaptic excitation from the dorsal quadrant.

3. Inhibition from motor axon collaterals was evoked with a latency (1·2–2·0 msec) suggesting a disynaptic linkage and had the same time course as in motoneurones. It prevented synaptic activation of 60% of interneurones and decreased the firing index and delayed generation of spikes in the remaining.

4. The interneurones with convergence of monosynaptic Ia excitation and inhibition from motor axon collaterals were found in the ventral horn dorsomedial to motor nuclei. No inhibition by antidromic volleys could be detected in interneurones located in intermediate nucleus and activated monosynaptically from Ia, Ib, group I or cutaneous afferents.

5. It was concluded that the ventral Ia interneurones inhibited by volleys in recurrent motor axon collaterals mediate the reciprocal Ia inhibition to motoneurones.

INTRODUCTION

Reciprocal inhibition of motoneurones from large muscle spindle (Ia) afferents is one of the most thoroughly analysed spinal reflex actions.

Originally it was believed that Ia afferents had direct effect on motoneurones (Lloyd, 1941), hence the term 'direct inhibition'. Later studies showed, however, that an interneurone must be interpolated in the Ia inhibitory pathway (Eccles, Fatt & Landgren, 1956; Eide, Lundberg & Voorhoeve, 1961; Araki, Eccles & Ito, 1960; Eccles, 1964). Eccles *et al.* (1956) proposed that these interneurones were located in the intermediary region, where they found cells monosynaptically activated from Ia afferents. Their properties conformed with then known properties of the Ia inhibitory pathway.

Recent studies of the reciprocal Ia inhibitory pathway have been focused on its control from other neuronal systems. Facilitation of transmission in the Ia inhibitory pathway was found from descending as well as segmental systems through their presumed excitatory action on the interneurones (Lundberg, 1970). The experiments described in a preceding paper (Hultborn, Jankowska & Lindström, 1971*a*) revealed that reciprocal Ia inhibition is under inhibitory control from motor axon collaterals, by all evidence through post-synaptic inhibition of the interneurones. This finding offered a new possibility for identifying the interneurones of the Ia inhibitory pathway from their pattern of convergence. Accordingly we have searched for interneurones with convergence of monosynaptic Ia excitation and inhibition from motor axon collaterals.

From studies of recurrent facilitation (disinhibition) of motoneurones it was already postulated that some interneurones inhibitory to motoneurones are subject to recurrent inhibition (Wilson, 1959; Wilson & Burgess, 1962*a*). Many investigators have found interneurones with inhibition from motor axon collaterals, although without identifying their excitatory afferent connexions (Frank & Fuortes, 1956; Hunt & Kuno, 1959; Koizumi, Ushiyama & Brooks, 1959; Wilson & Burgess, 1962*b*; Biscoe & Krnjević, 1963; Smerdlov & Maksimova, 1965; Willis & Willis, 1966).

Convergence of excitation from group Ia afferents and inhibition from motor axon collaterals has now been found in a group of interneurones located in the ventral horn dorsomedial to the motor nuclei. A brief preliminary report of some of the results has been published (Hultborn, Jankowska & Lindström, 1968).

METHODS

Preparation. The experiments were performed on eleven cats spinalized and anaemically decorticated as described by Andén, Jukes, Lundberg & Vyklický (1966). The interneurones were searched for in L 5, L 6 and L 7 spinal segments. The ventral roots L 5–S 1 were transected ipsilaterally and their central parts were mounted for stimulation. Caudal to the transection in the lower thoracic level the spinal cord was split into two spinal halves at a length of about 2 cm. The ipsilateral half was further

subdivided into dorsal and ventral fascicle. The dissection and maintenance of the preparation were described previously (Hultborn *et al.* 1971*a*).

Stimulation and recording. In order to analyse afferent connexions to the interneurones a number of peripheral nerves from the ipsilateral and contralateral hind limbs were stimulated electrically. Single and tetanic stimuli were used. Recording was made with glass micropipettes filled with 2 M-K-citrate solution (resistance 1·5–6·0 MΩ, tip diameter 1·0–1·5 μ). Otherwise recording and stimulation were as described previously (Hongo, Jankowska & Lundberg, 1966; Hultborn *et al.* 1971*a*).

Criteria for identification of interneurones. Interneurones were differentiated from cells giving origin to ascending tracts and from motoneurones by the absence of antidromic invasion from the dissected spinal halves as well as from the ventral roots (Hongo *et al.* 1966).

In the case of interneurones with a convergence of monosynaptic Ia excitation and inhibition from motor axon collaterals, i.e. with an input similar to motoneurones, the differentiation from the latter was of particular importance. The antidromic invasion of motoneurones recorded intracellularly may be blocked, so that only a small M spike is evoked (Brock, Coombs & Eccles, 1953). The spike, as well as small extracellular antidromic spikes, may be difficult to see on the background of big motor field potentials. Therefore the stimulation of the ventral roots was graded so that any antidromic responses of all-or-none character could be detected. When recording extracellularly the orthodromically evoked monosynaptic spikes were in addition preceded by a maximal stimulus to the ventral roots with a time interval so short that if an antidromic spike was evoked by the antidromic volley, the response to the orthodromic volley would fall within the refractory period and be obliterated. Tested in this way the orthodromic responses of motoneurones were abolished (Fig. 1*G*) while those of the cells classified as interneurones were not affected (Fig. 1*C*). With intracellular recording, stimulation of the ventral roots was combined with a depolarization of the cells through the recording electrode in order to overcome a possible block at the axon–soma junction (Eccles, 1953).

The interneurones were differentiated from motoneurones also by their ability to follow high frequency of peripheral stimulation and their lack of reciprocal Ia inhibition.

Motoneurones can follow orthodromic stimulation only to 50–100/sec (Eccles & Rall, 1951; Eccles, 1953), while interneurones in the Ia inhibitory path are supposed to follow much higher frequencies (cf. Eccles *et al.* 1956) and the discharge frequencies of interneurones excited by stretch of the muscles may be of the same order as of afferent fibres (Kolmodin, 1957). The graph in Fig. 1 gives a comparison of frequency following of twelve motoneurones and of fourteen cells which were not invaded antidromically and hence classified as interneurones. The ordinate gives numbers of spikes evoked within 50 msec and the abscissa the stimulation frequencies. At the frequencies higher than 150/sec the motoneurones fired only a few spikes at the beginning of stimulation (Fig. 1*H*) while all the interneurones were able to follow stimulus frequencies over 300/sec (up to 400–500/sec when checked) showing a distinct difference between the two kinds of cells. Examples of high-frequency interneuronal responses are given in Figs. 1*E*, 2*L* and 3*F*.

The Ia IPSPs are evoked in virtually all motoneurones to several muscles (e.g. to Q, PBSt, Grac, DP; R. M. Eccles & Lundberg, 1958*a*). Lack of group Ia reciprocal inhibition in cells with monosynaptic connexions from Q, PBSt, Grac and DP was therefore considered as a supplementary criterion that the cell was not a motoneurone. The presence of this inhibition does not indicate the opposite, since a weak inhibition from Ia afferents from antagonists was seen in some cells which by other criteria could be classified as interneurones. Fig. 1*A*, *B* and *E*, *F* show different

effects of conditioning stimulation of Ia afferents from antagonists in an interneurone and in a motoneurone.

The main results presented in this paper are based on an investigation of sixty-nine cells inhibited by volleys in recurrent motor axon collaterals. Forty-one cells, referred to as group A cells, were classified as interneurones with the greatest confidence. They were not invaded antidromically either from the ventral roots or from the spinal tracts, followed high frequency of stimulation (at least 300/sec) and/or were not inhibited from antagonists. In the remaining twenty-eight cells, group B cells,

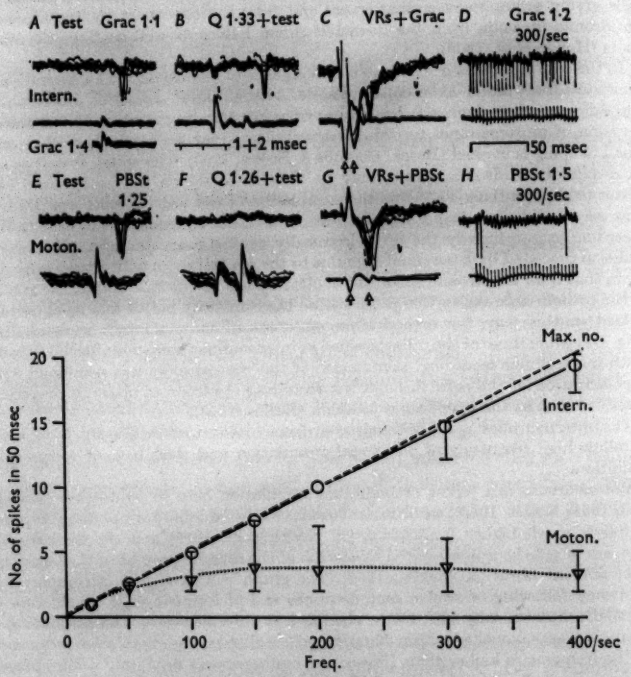

Fig. 1. For legend see opposite page.

the differentiation from motoneurones was investigated less thoroughly than in group A. They were not invaded antidromically, but it is unknown if they fulfilled all the other criteria. About 100 interneurones not inhibited from recurrent motor axon collaterals were investigated for comparison.

Location of interneurones. In each region of the spinal cord investigated, an electrode was left in the last track and its position was verified after fixation of the spinal cord in 10 % formalin and embedding in paraffin. The position of each interneurone could be determined in respect to the track in which the electrode was left, as in each region all the tracks were parallel and at a known distance from each other.

The depth at which the interneurones were located was calculated from the known distance from the surface of the cord dorsum (from micromanipulator readings) and the coefficient of shrinkage of the spinal cord. The estimated error in depth reading (from comparing several readings for contact with the cord dorsum) was about 50 μ. The coefficient of shrinkage of the spinal cord due to fixation and histological procedures was determined from the change in distance in the frontal plane between two parallel electrodes, 2 mm apart, introduced in the spinal cord before the animal was sacrificed. The composite Figures (Fig. 4) summarizing location of interneurones found in different experiments were made as follows. The location of the individual interneurones was marked on drawings from the corresponding transverse sections of the spinal cord. These drawings were then put together so that the contours of the grey matter and the extent of motor nuclei would fit, separately for L 7 and L 6 segmental levels. Special care was taken to define location of the interneurones in respect to the motor nuclei. The extent of the latter was determined, from Nissl stained sections, as the area at which the motoneurone somas were seen. In the last experiments few cells were marked by intracellular injection of a fluorescent dye (Procion Yellow; Stretton & Kravitz, 1968) and their location could be determined more precisely.

Abbreviations. The following abbreviations are used: anterior biceps (AB), anterior biceps and semimembranosus (ABSm), deep peroneus branches to tibialis anterior and extensor digitorum longus (DP), flexor digitorum and hallucis longus (FDL), gracilis (Grac), popliteus (Popl), posterior biceps (PB), posterior biceps and semitendinosus (PBSt), sartorius (Sart), semimembranosus (Sm), semitendinosus (St),

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Legend to Fig. 1.

Tests used for differentiating interneurones from motoneurones. In *A–H* upper traces are extracellular records from an interneurone (upper row) and a motoneurone (lower row). Lower traces are cord dorsum potentials. The lowermost trace in *A* shows incoming afferent volley from Grac evoked by stimulation maximal for group I afferents. *A* and *E*, monosynaptic responses evoked by stimulation of peripheral nerves. *B* and *F*, effects of conditioning stimulation of group Ia afferents from antagonistic muscles. *C* and *G*, effects of conditioning stimulation of the ventral roots. The first and second arrows indicate the antidromic and orthodromic volleys respectively (note that the motoneurone fired only one spike). *D* and *H*, single trace records of the responses to a tetanic stimulation of afferent nerves.

The graph gives mean values of maximal numbers of spikes (ordinate) evoked within 50 msec periods by tetanic stimulation with different frequencies (abscissa). The stimulus strength was maximal for group I afferents. The lower and the upper curves are for twelve motoneurones (six with intra- and six with extracellular recording) and fourteen interneurones (four with intra- and ten with extracellular recording) respectively. The extreme ranges are given by vertical lines. The interrupted line shows the maximal possible number of responses within the period of measurement. Time calibration for *A–C* and *E–G* is in *B* and for *D* and *H* in *D*.

In this and in the following figures positivity in micro-electrode recording and negativity in cord dorsum recording are signalled upwards. The strengths of the stimulation are expressed in multiples of thresholds of the nerves or ventral roots.

tibialis (Tib), tensor fasciae latae (TFL), quadriceps (Q), dorsal and ventral roots (DR and VR) dorsal and ventral quadrants of the spinal cord (DQ and VQ), excitatory, inhibitory and recurrent inhibitory post-synaptic potentials (EPSP, IPSP and RIPSP), flexion reflex afferents (FRA), firing index (FI), ipsi- and contralateral (i and co).

RESULTS

Interneurones monosynaptically activated from group I a afferents and inhibited from motor axon collaterals

The recurrent inhibitory effects in the interneurones were judged from: (1) presence of the recurrent IPSPs, (2) arrest of resting discharges and (3) interference with the synaptic activation from primary afferents. All

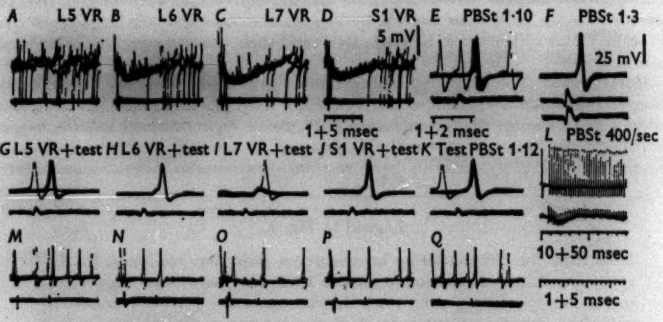

Fig. 2. Recurrent inhibition of resting discharge and of monosynaptic spike potential in an interneurone. Upper traces are intracellular records from an interneurone in rostral L 7. Lower traces are cord dorsum potentials. *E* and *F*: spike potentials evoked by stimulation of the nerve to PBSt with a strength submaximal and maximal for I a afferents respectively. The lowermost record in *F* shows the amplitude of the incoming volleys evoked by stimulation maximal for I b afferents. *A–D*: effects of stimulation of L 5, L 6, L 7 and S 1 VRs on resting discharge of the interneurone and the RIPSPs evoked by this stimulation. *G–J* and *M–P*: effects of stimulation of the same ventral roots on initiation of the monosynaptic spike potential. Corresponding records in *G–J* and in *M–P* were taken simultaneously with a fast and a slow sweep speed, the fast records showing an expanded part of the slow records. Voltage calibrations are for *A–D* in *D* and for all the other records in *F*. Time calibrations are for *A–D* in *D*, for *E–K* in *E*, for *L* in *L* and for *M–Q* to the right of *Q*.

these effects can be seen in the cell illustrated in Fig. 2. The cell was monosynaptically excited by stimulation of the nerve to PBSt at a strength submaximal for I a afferents (cf. the amplitudes of the incoming volleys in *E* and *F*). The cell followed stimulation frequencies of 400/sec over a period of nearly 100 msec. Recurrent inhibition was evoked from the L 6, L 7 and

S1 VRs and the recurrent IPSPs from these roots are shown at higher gain in A–D. The largest IPSP (*C*) and the most effective inhibition of the resting discharge (*C*, *O*) was evoked from the L7 ventral root. Records in *G–J* and *M–P* show the effects of conditioning stimulation of the ventral roots on the synaptic firing evoked from PBSt, with the control response given in *K* and *Q*. Stimulation of the L7 VR almost completely prevented the initiation of the spikes. Stimulation of the L6 and S1 VRs was less effective and merely delayed spike initiation. No effect could be detected from the L5 VR.

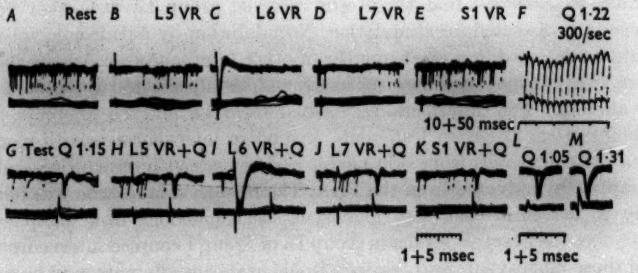

Fig. 3. Recurrent inhibition of resting discharge and of monosynaptic spike potential in an interneurone. Upper traces are extracellular records from an interneurone in caudal L6. Lower traces are cord dorsum records. *L* and *M*, the spike potential evoked by a submaximal (*L*) and approximately maximal (*M*) stimulation of group Ia afferents from Q. *A*, resting discharge of the cell. The effects of stimulation of L5–S1 VRs on the resting discharge of the cell are shown in *B–E* and on the monosynaptic spike potential in *H–K*. Time calibration in *F* is for *F*, in *K* for *A–E* and *G–K*, in *L* for *L* and *M*.

In Fig. 3 are extracellular records from another interneurone which was monosynaptically activated from group Ia afferents from Q. The separation between the Ia and Ib volleys (Bradley & Eccles, 1953: Eccles, Eccles & Lundberg, 1957) was poor in this case but comparison of cord dorsum potentials taken at higher amplification and faster sweep speeds (not illustrated) suggested that the Ia volley in *M* was maximal. The discharge in *L* was thus evoked at a strength submaximal for Ia afferents. The recurrent inhibition was evidenced by a pause in resting firing of the interneurone (*A–E*) and by the interference with the group Ia evoked spike (*G–K*). The spike was completely inhibited when preceded by stimulation of the L6 VR. Weaker recurrent inhibition was evoked from the L5 and L7 VRs, and probably none from the S1 VR.

The records of Figs. 2 and 3 show that the inhibition could be evoked from recurrent motor axon collaterals of more than one ventral root (cf. below). Therefore a synchronous stimulation of several ventral roots was used when the strongest possible recurrent actions were to be determined and, in case of negative results, in order to ascertain the absence of recurrent inhibition.

Monosynaptic Ia excitation was found in sixty-seven of sixty-nine cells with recurrent inhibition from motor axon collaterals (forty-one belonging to group A and twenty-six to group B, cf. Methods). The remaining two interneurones were polysynaptically activated from the FRA (cf. below) but it cannot be excluded that these cells received group Ia excitation from nerves not dissected for stimulation. No inhibition by antidromic volleys could be detected in a great number of other interneurones. Among them were twenty-nine interneurones monosynaptically excited by volleys in Ia afferents, fifty interneurones excited from Ia and Ib afferents, Ib afferents alone or group I afferents, seventeen interneurones with monosynaptic connexions from skin nerves and seventeen interneurones polysynaptically activated from the FRA. The recurrent inhibition was fairly easy to detect in cells with monosynaptic connexions from periphery. Therefore the negative results in group Ia or group I coupled interneurones and those monosynaptically excited from cutaneous afferents may be considered as significant although obtained in relatively small samples of cells. In the case of interneurones polysynaptically activated from the FRA the inhibition was more difficult to detect and cannot be completely ruled out in some cells.

A comparison between Ia activated interneurones with and without recurrent inhibition did not reveal any other striking difference in their functional properties. The two types of Ia interneurones are illustrated in Fig. 4. Nearly maximal EPSPs (*C* and *I*) were evoked at strengths submaximal for Ia afferents, with no further increase in amplitude with activation of Ib afferents (cf. *D* to *E* and *J* to *K*). In both types of cell the EPSP could be graded (as shown in *A–D* and *G–J*, Fig. 4) and spike discharges could be evoked by very small Ia volleys.

Location of Ia interneurones with and without inhibition from motor axon collaterals

Terminal branches of Ia primary afferent collaterals have been found in three regions of the spinal grey matter: (i) within motor nuclei, (ii) in the intermediate nucleus, and (iii) just dorsomedial to motor nuclei (Szentágothai, 1967). The interneurones with a convergence of monosynaptic Ia excitation and inhibition from motor axon collaterals were therefore searched for in all these three regions. Interneurones monosynaptically

excited from Ia afferents were previously found in the intermediate nucleus (Eccles, Fatt, Landgren & Winsbury, 1954; Eccles *et al.* 1956; Eccles, Eccles & Lundberg, 1960; Hongo *et al.* 1966), and within the motor nuclei (A. Lundberg, personal communication). Interneurones inhibited by antidromic volleys were previously encountered mostly in the ventral horn (cf. Wilson & Burgess, 1962*b*; Willis & Willis, 1966).

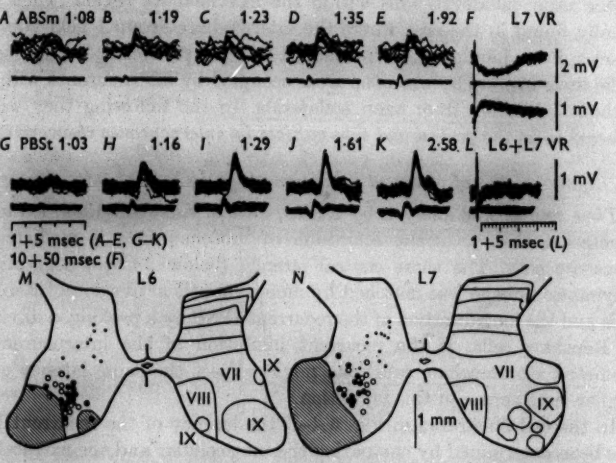

Fig. 4. Location of cells with convergence of Ia excitation and of recurrent inhibition. Upper traces in *A–F* and *G–L* are intracellular records from two interneurones located dorso-medially to the motor nuclei in caudal L6 (*A–F*) and in the intermediate nucleus in rostral L7 (*G–L*) respectively. Lower traces are cord dorsum potentials. The lowermost trace in *F* is the extracellular potential recorded when the electrode was just outside the cell (note higher amplification of the extracellular than of the intracellular records). Voltage calibrations are for the micro-electrode recording. The records in *F* and *L* were taken with time constant 0·8 sec. *M* and *N*, location of the interneurones monosynaptically excited by Ia afferents. Filled (●) and open (○) circles denote group A and group B interneurones inhibited from motor axon collaterals. Points (.) denote the interneurones not inhibited. The hatched areas indicate the extent of motor nuclei. The right sides of the diagram show Rexed's (1954) laminae. Further explanation in the text.

Fig. 4*M* and *N* show the location of interneurones monosynaptically excited by volleys in Ia afferents, with (filled and open circles) and without (points) inhibition from motor axon collaterals. These composite figures were made by superposition of drawings from separate experiments, as described under Methods. The location of the group B interneurones (open

circles) has been indicated separately because their differentiation from motoneurones was less thorough than of group A interneurones (filled circles). All cells inhibited by antidromic volleys were located dorsomedially to motor nuclei, in the ventral part of Rexed's (1954) lamina VII. An antidromic motor field potential often could be recorded in their proximity (cf. Figs. 3, 7–9). The location of Ia interneurones not inhibited from motor axon collaterals falls within the intermediate region. They were usually found in the area where the intermediate group I field potential (Eccles, Fatt, Landgren & Winsbury, 1954) was pronounced. There seems to be little overlap between the areas occupied by interneurones with and without inhibition from axon collaterals. In the following they will be referred to as the *ventral and intermediate Ia interneurones* respectively.

Characteristics of recurrent inhibition of the Ia excited interneurones

Time course. The analysis by Eccles, Fatt & Koketsu (1954) led to the conclusion that recurrent inhibition of motoneurones is mediated by Renshaw cells. The short central latency (below 2 msec) suggesting a disynaptic linkage was matched by monosynaptic activation of Renshaw cells and the long duration of the recurrent IPSP by a prolonged discharge in Renshaw cells. If the recurrent inhibition of the interneurones is mediated via Renshaw cells, one might expect the same latency and a similar time course of this inhibition.

In the cell illustrated in Fig. 5*A–D* the latency of the recurrent IPSP has been determined by comparing the intracellular and the extracellular records (*A*, superimposed in *C*), as well as the intracellular records of the RIPSP, before and after its reversal during a hyperpolarization of the cell (*B*, superimposed in *D*). The central latency (1·3 msec) was measured from the time of arrival of the antidromic volley (first arrows in *C* and *D*) and the points of divergence of the superimposed records (second arrows). In other cells the central latencies of the RIPSPs were between 1·2 and 2·0 msec. These latencies are within the same range as the latencies of RIPSPs in motoneurones and indicate a disynaptic linkage between motor axon collaterals and Ia interneurones.

Graphs *E* and *F* give the time course of the recurrent inhibition judged from the effects of the conditioning stimulation of the ventral roots on the monosynaptic spike potential of an interneurone recorded extracellularly. The measure of recurrent inhibition was the firing index of the cell (ordinate), plotted against the time interval between the arrival at the spinal cord of the antidromic and orthodromic incoming volleys (abscissa). The firing index started to decrease at conditioning-testing interval of about 1.2 msec. In similar measurements in other cells the central latencies of the inhibitory effects were between 0·3 and 1·2 msec. The total duration of

the recurrent inhibition in the interneurones was about 50–80 msec. The strongest inhibitory effects were evoked within conditioning-testing intervals of 5–20 msec.

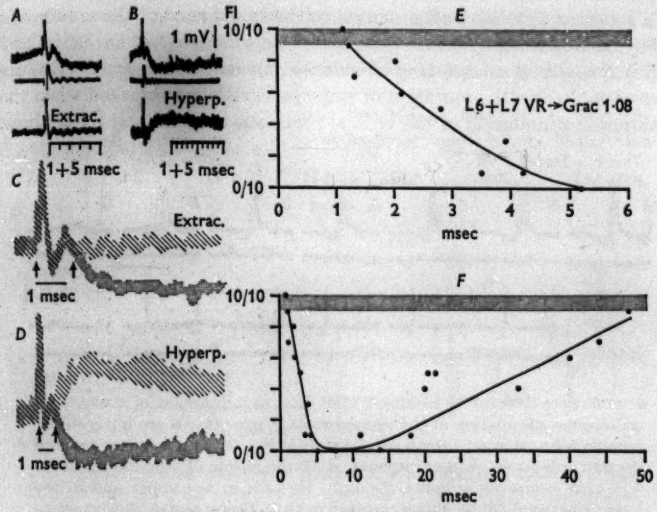

Fig. 5. Time course of recurrent inhibition of two interneurones. Upper traces in *A* and *B* are intracellular records from an interneurone. Middle traces are cord dorsum potentials. Lower trace in *A* is an extracellular potential and in *B* a reversed IPSP recorded during passage of the hyperpolarizing current. The records in *A* and *B* are superimposed in *C* and *D* respectively. The first arrows indicate time of arrival of the antidromic volleys to the spinal cord. The second arrows show the divergence points of the superimposed traces. *E* and *F*, time course of recurrent inhibition of the monosynaptic spike potential evoked in an interneurone (the same as in Fig. 1). Ordinate: firing index (F1) of the cell. Abscissa: time intervals between arrival to the spinal cord of the incoming volleys evoked by the conditioning and by the test stimulation. Dotted area: firing index of the cell when the test stimulation was used alone and line when it was preceded by the conditioning stimulation of the ventral roots.

Effectiveness of the recurrent inhibition of the interneurones

The effectiveness of the recurrent inhibition in interfering with synaptic activation of the interneurones depended on several factors.

(i) *Convergence from ventral roots.* In most of the interneurones the recurrent inhibition from motor axon collaterals was evoked from two and sometimes from three ventral roots (cf. Figs. 2 and 3). The synchronous

stimulation of different ventral roots revealed a spatial summation of their effects; it could completely prevent initiation of spikes in the interneurones, even if inhibition from single ventral roots was only partial.

(ii) *Conditioning–testing* interval. The most effective recurrent inhibition occurred 5–20 msec after arrival to the spinal cord of the antidromic volley, as described above.

(iii) *Strength of conditioning stimulation.* In cases of very effective recurrent inhibition the initiation of spike potentials was blocked when the antidromic stimulation of the ventral roots was submaximal for α-fibres.

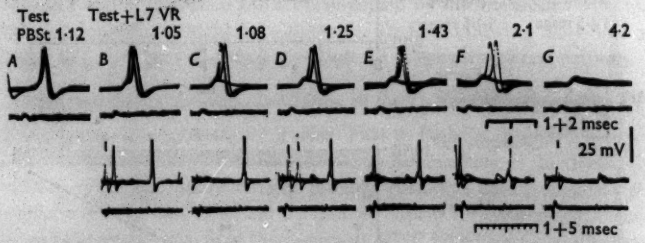

Fig. 6. Effectiveness of recurrent inhibition as a function of strength of antidromic stimulation of the ventral roots. Upper traces are intracellular records from an interneurone (the same as in Fig. 2). Lower traces are cord dorsum potentials. *A*, test response. *B–G*, the effects of stimulation of L7 VR with increasing stimulus strength. Records in the upper and in the lower row were taken simultaneously with a faster and a slower sweep speed.

When the inhibitory effects were weaker maximal inhibition was reached within stimulus strength maximal for α-fibres as shown in Fig. 6*B–G*. The test orthodromic stimulation of PBSt (*A*) was constant, while the strength of the conditioning stimulation of the L7 VR was increased from *B* to *G* (cf. lower traces in slow speed records). In this interneurone the initiation of the spikes was blocked completely only when the conditioning stimulation was maximal for α-fibres. A submaximal stimulation decreased the firing index of the cell and the spikes were generated with increased and varying delays. This resulted in a marked asynchrony of the firing. Following stimulation of the ventral roots the spikes were generated within 0·40–0·60 msec after the onset of the EPSP. Without the conditioning stimulation they appeared within 0·25–0·35 msec. A similar delay in generation of the spikes was observed also in other interneurones.

(iv) *Strength of orthodromic stimulation.* When the monosynaptic spike potentials were evoked at near threshold stimulation of the Ia afferents, their initiation could be prevented in almost all the interneurones. When stronger stimulation was used, submaximal or maximal for group Ia

afferents, the effects of conditioning stimulation of the ventral roots varied in different cells. This was tested when all the ventral roots were stimulated at strengths supramaximal for α-efferents and at optimal conditioning–testing intervals. In 60% of the interneurones recurrent inhibition could prevent generation of the spikes independently of the strength of the test orthodromic stimulation. In the remaining cells it only reduced their firing index and increased the delay in generation of the

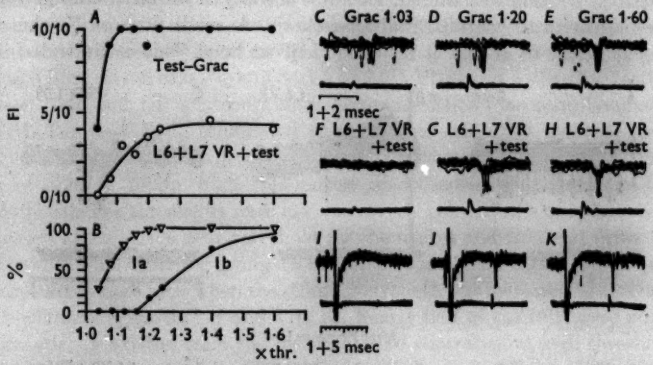

Fig. 7. Effectiveness of recurrent inhibition as a function of strength of test stimulation. *A*: firing index of an interneurone (ordinate) plotted against different strength of orthodromic stimulation (abscissa), when test stimulation was used alone (upper curve) and when it was preceded by a conditioning stimulation of the ventral roots (lower curve). The conditioning stimulation of the ventral roots was constant. *B*: amplitudes of the Ia and Ib incoming volleys (in % of their maximal amplitudes). *C–E* and *F–K*: sample records for curves in A. The corresponding records in *F–H* and *I–K* were taken simultaneously with a fast and a slow sweep speed. Time calibration in *C* is for *C–H* and in *I* for *I–K*.

spikes. An example of such an interneurone is given in Fig. 7. In this case the conditioning stimulation was constant while the strength of the test stimulation was increased. *B* shows the amplitudes of group Ia and Ib incoming volleys evoked by different strengths of stimulation of the nerve to Grac. The corresponding responses of the cell are plotted in *A* and illustrated by the sample records *C–K*.

Characteristics of activity of the interneurones and their connexions

Rhythmic activity. Nearly all the Ia interneurones inhibited by volleys in motor axon collaterals displayed tonic activity similarly to the interneurones found by Hunt & Kuno (1959), Wilson & Burgess (1962*b*), Biscoe & Krnjević (1963), Smerdlov & Maksimova (1965) and Willis &

Willis (1966). They usually fired with a sustained, rather irregular, rate though in some cells high-frequency bursts were separated by long silent periods. The average firing frequency varied considerably in different cells. Usually it was between 20 and 60/sec, but in some cells frequencies up to 100–110/sec were observed. In extracellular recording injury firing of the cells usually could be excluded since the firing rates of the cells were constant over fairly long periods of time and did not change when the microelectrode position was altered. The tonic activity of the interneurones was very sensitive to pentobarbital anaesthesia. A small dose of Nembutal was sufficient to abolish it for about half an hour. This is illustrated in

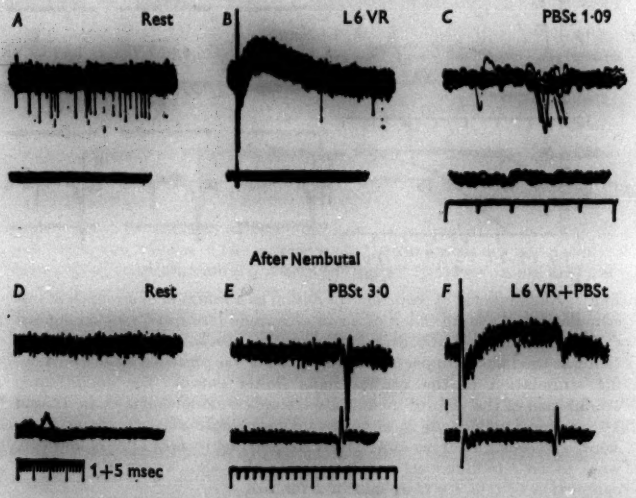

Fig. 8. Effects of Nembutal on Ia coupled interneurones. Upper traces are extracellular records from an interneurone with monosynaptic connexions from Ia afferents from PBSt. Lower traces are cord dorsum potentials. *A–C*, before injection of Nembutal. *D–F*, after injection of Nembutal, 6 mg/kg. Time calibration in *C* is for *C*, in *D* for *A*, *B* and *D*, in *E* for *E* and *F*. Further explanation in the text.

Fig. 8 which shows extracellular records from an interneurone with a resting discharge (*A*) abolished completely (*D*) by Nembutal (6 mg/kg). This was accompanied by an increase in threshold for initiation of the spike potential from Ia afferents. However, when evoked, the spike was as effectively inhibited from motor axon collaterals (*E–F*) as before.

Peripheral connexions. A full analysis of synaptic connexions to the ventral Ia interneurones would require intracellular recording from a large

number of cells and was beyond the aim of the present investigation. The present material consists of intracellular records from seventeen cells which were fully analysed for effects from all nerves dissected. It is supplemented by extracellular records from sixteen cells.

The characteristic feature of synaptic connexions to the interneurones seems to be their narrow monosynaptic excitatory input from group Ia afferents from one or a few muscles, mainly synergists. A limited convergence of monosynaptic excitation was observed in one-third of the interneurones, some of them recorded intracellularly, and some extracellularly. The convergence was from group I, probably group Ia afferents from Q and ABSm (seven cells), Q and Sart (one cell), PBSt and ABSm (two cells), Sart, Grac and DP (one cell), Sart, Grac and PBSt (one cell), Grac and FDL–Tib. post–Popl, stimulated together (one cell). The monosynaptic linkage from group Ia afferent seems to be strong since the interneurones were able to follow high frequencies of orthodromic stimulation (cf. Methods). The thresholds were low – in several cells EPSPs or spikes were evoked at strengths near threshold for the nerves and maximal firing by stimuli submaximal for group Ia afferents. Usually no additional excitatory effects were seen when the stimulus strength was increased to activate Ib efferents (Figs. 4*D–E* and 7*A*, B). Nearly half of the cells were polysynaptically excited from the ipsilateral and contralateral high threshold muscle and skin afferents (FRA; R. M. Eccles & Lundberg, 1959). The excitation from the ipsilateral FRA (cf. Fig. 9 *G–I*) was evoked in interneurones with monosynaptic Ia excitation from either flexors or extensors. Excitation from the contralateral FRA was found in the same cells which were excited from the ipsilateral FRA; the contralateral actions were usually weaker than the ipsilateral ones.

Inhibitory effects were also found but only those evoked by volleys in Ia afferents from antagonists were analysed systematically (cf. Methods and Fig. 1). An IPSP from group I (probably Ia) afferents was observed once, and was evoked in a Q interneurone from the nerve to Sart. In the cells recorded from extracellularly a weak decrease of firing index by single shocks to antagonists was observed in one PBSt interneurone and, when trains of shocks were used, in two PBSt and in one Q interneurone. In the latter case the conditioning–testing intervals were less than 10 msec (from the first shock) so that it is rather unlikely that the effect was caused by presynaptic inhibition. Inhibition (IPSP in intracellular recording or arrest of resting discharge in extracellular recording) was also found from the ipsilateral FRA. This effect must be, however, considered with caution since it cannot be excluded that inhibition was secondary to excitation of motoneurones. However, in one cell the FRA IPSPs from several nerves were larger than the maximal RIPSP which

suggests that at least part of the former was not due to activation of motoneurones and the recurrent loop.

Descending connexions. Facilitation of reciprocal Ia inhibition can be evoked from cortico-, rubro- and vestibulospinal pathways (Lundberg & Voorhoeve, 1962; Grillner, Hongo & Lund, 1966, 1968; Hongo, Jankowska & Lundberg, 1969). In the case of the effect mediated by vestibulospinal tract fibres the very short latency of the facilitation leaves no doubt that it is evoked through monosynaptic excitatory action on the interneurones mediating Ia inhibition (Grillner *et al.* 1966, 1968; cf. Lundberg, 1970). As the vestibulospinal tract descends in the ventral part of the spinal cord (Nyberg-Hansen & Mascitti, 1964) it was of special interest to investigate synaptic effects from stimulation of the dissected ipsilateral ventral quadrant.

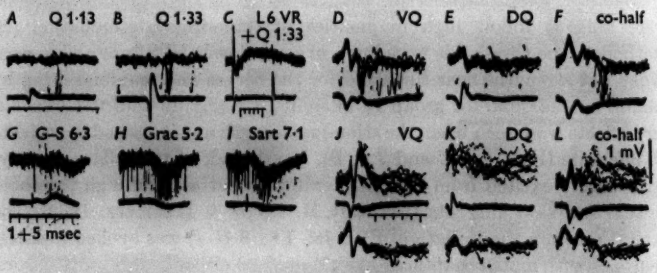

Fig. 9. Peripheral and descending connexions. In *A–I* the upper traces are extracellular records from an interneurone with Ia connexions from Q and the lower traces are the cord dorsum potentials. In *J–L* the upper traces are intracellular records from another Q interneurone, the lower traces are extracellular potentials recorded after withdrawal of the microelectrode to a just extracellular position (note the faster sweep of the extracellular records) and the middle traces are the cord dorsum potentials. Time calibrations (1 + 5 msec) are in *A* for *A*, *B*, *D–F* and extracellular records in *J–L*, in *G* for *G–I*, in *J* for *J–L*. Voltage calibration is for microelectrode recording in *J–L*.

Fig. 9*D* and *J* give examples of short latency responses from ipsilateral ventral quadrant in two different Ia interneurones. The segmental latency of the extracellular spike in *D* is 0·6 msec when measured from the peak of the first positive wave. A comparison of the intracellular record of the EPSP in *J* with the field potential recorded just outside the cell gives a segmental latency of 0·7–0·8 msec. EPSPs, or spikes with a segmental latency of less than 1·0 msec, were evoked by stimulation of the ventral quadrant, or the whole ipsilateral spinal half, in two of four Q interneurones and in two of three ABSm interneurones (intracellular

records), as well as in three interneurones recorded from extracellularly, which were monosynaptically excited from Q, G-S and PBSt respectively.

The shortest latency post-synaptic potentials or spikes from the ipsilateral dorsal quadrant of the spinal cord and from the contralateral spinal half had latencies suggesting a disynaptic linkage (between 1·0 and 2·0 msec). Such effects were observed in four cells.

Rostrocaudal distribution of the interneurones. In the case of the reciprocal Ia inhibition between Q and PBSt the Ia afferents may enter the spinal cord 1 or 2 segments away from the motoneurones inhibited by them (Eccles *et al.* 1956). This anatomical arrangement gives an opportunity to investigate the location of the Ia interneurones in relation to the primary afferents which excite them, to motoneurone axon collaterals from which they are inhibited and to motoneurones which they are supposed to inhibit. In the present study the L5, L6 and L7 spinal segments were explored in search for the Ia interneurones inhibited by antidromic volleys. Twenty-nine of thirty-one interneurones excited from Q were found in the L5–L6 and two in the L7 segments. Of ten interneurones with monosynaptic connexions from PBSt nine were located in the L7 and only one in the L6 segment. The location of these interneurones was thus closer to the entrance level of their excitatory afferents than to the level at which are located motoneurones innervating antagonists.

The interneurones could be inhibited by stimulation of one to three ventral roots. These roots were different for cells with different peripheral connexions. Q, Sart and Grac interneurones were most effectively inhibited from the L5 and L6 VRs (cf. Fig. 3), ABSm interneurones from the L6 and L7 VRs and PBSt interneurones from the L7 and S1 VRs (cf. Fig. 2). The most effective inhibition was therefore evoked from the segments at which are located motoneurones to the muscles supplying Ia excitation to the interneurones.

DISCUSSION

Interneurones monosynaptically activated by Ia afferents from muscle spindles and inhibited by volleys in recurrent motor axon collaterals have been found in a region dorsomedial to motor nuclei, corresponding to a semilunar area between the intermediate and motor nuclei, where Szentágothai (1967) found terminal arborization of collaterals of Ia afferents. No recurrent inhibition could be detected of Ia interneurones in the intermediate nucleus. These findings cast doubt on the original hypothesis that inhibition from Ia afferents to motoneurones is mediated by Ia activated interneurones in the intermediate region (Eccles *et al.* 1956; Eccles, 1969). In reconsidering this hypothesis and discussing the role of the ventral Ia interneurones recurrent inhibition from motor axon collaterals

is an important, but not the sole, criterion. However, it will be shown that the ventral Ia interneurones possess also other properties required of Ia inhibitory interneurones.

Postulated properties of Ia inhibitory interneurones. Characteristics of interneurones in the Ia inhibitory pathway to motoneurones have been inferred from studies of transmission in the pathway. One approach has been to define the properties of the synaptic excitatory relay from Ia afferents to the inhibitory interneurones (Eccles *et al.* 1956). The other main approach is represented by the attempts at defining the convergence on the interneurones from Ia afferents and from other neuronal systems (Lundberg, 1969, 1970). The results of these studies indicate that the Ia inhibitory interneurones are characterized by

I. Monosynaptic excitation from Ia afferents.

II. Low threshold for excitation (Bradley & Eccles, 1953; Eccles, Eccles & Lundberg, 1957) which, at least under anaesthesia, requires summation (R. M. Eccles & Lundberg, 1958*b*).

III. Capability to follow high-frequency peripheral stimulation (Bradley *et al.* 1953, cf. Eccles *et al.* 1956).

IV. Limited excitatory convergence from group Ia afferents from one muscle or a few related muscles (Lloyd, 1946; R. M. Eccles & Lundberg, 1958*a*, *b*).

V. Excitation by volleys in the FRA (Bruggencate, Burke, Lundberg & Udo, 1969; Lundberg, 1970).

VI. Excitation by volleys in descending tracts (Lundberg & Voorhoeve, 1962; Grillner *et al.* 1966; 1968; Hongo *et al.* 1969; cf. also Lundberg, 1969, 1970).

VII. Disynaptic inhibition by volleys in recurrent motor axon collaterals (Hultborn *et al.* 1971*a*).

Do the Ia interneurones in the ventral horn have the properties required to mediate reciprocal Ia inhibition? The interneurones inhibited by antidromic volleys in motor axons possess the first three of the required properties (I–III).

The convergence of Ia afferents (IV) onto them is from a fairly limited number of muscles, usually close synergists or those which contribute to the Ia IPSPs evoked in the same motor nuclei. For instance, the convergence of Ia afferents from Q and ABSm and from PBSt and ABSm which have different actions, corresponds with the origin of Ia IPSPs in Sart motoneurones (from Q and ABSm; R. M. Eccles & Lundberg, 1958*a*) and in TFL motoneurones (from PBSt and ABSm; Hongo *et al.* 1969). A wider convergence from group I afferents was seen in only three of thirty-three cells.

Excitation from the FRA (V) was evoked in many interneurones but at present it is difficult to compare these effects with the results from investi-

gations on the Ia inhibitory pathway. Bruggencate *et al.* (1969) concluded that volleys in the contralateral FRA have excitatory action on interneurones of the Ia inhibitory pathway to flexors. Correspondingly it has been suggested that interneurones to extensors receive excitatory action from the ipsilateral FRA (Lundberg, 1970). In our material there was no evident pattern in the effects from the ipsilateral and contralateral FRA; several of the ventral Ia interneurones were in fact excited from both limbs. For a correlation between the two effects it will be necessary not only to obtain a more detailed knowledge of the actions from the FRA on the Ia inhibitory pathways and the ventral Ia interneurones but also, in each case, to relate these effects to the pattern of reflex actions evoked from the FRA in motoneurones (Eccles & Lundberg, 1959).

Descending effects (VI) have so far only been investigated with stimulation of spinal tract fibres. It is of particular interest that monosynaptic EPSPs from the ipsilateral ventral quadrant of the spinal cord are evoked in interneurones excited by volleys in Ia afferents from extensors. These monosynaptic EPSPs, recently found in ventral group Ia interneurones also by Skinner, Willis & Hancock (1970), may well originate from the vestibulospinal tract fibres, which selectively facilitate transmission in the Ia inhibitory path from extensors to flexors by a presumed monosynaptic excitatory action on the interposed interneurones (Grillner, Hongo & Lund, 1966; cf. Lundberg, 1970). Stimulation of well-defined descending systems is needed to investigate whether the Ia interneurones in the ventral horn are subject to all the postulated descending actions.

The inhibition of the interneurones by volleys in recurrent motor axon collaterals (VII) has been described in detail under Results. It remains to compare characteristics of this inhibition with the recurrent depression of transmission in the Ia inhibitory pathway to motoneurones (Hultborn *et al.* 1971*a*).

There is a good correspondence between the time course of the recurrent inhibition of Ia interneurones and the depression of Ia IPSPs. The segmental latency of inhibition in interneurones suggests a disynaptic pathway from the recurrent motor axon collaterals to interneurones. Their monosynaptic activation should be thus prevented by antidromic volleys preceding afferent volleys by a time interval as short as about 0·5 msec (corresponding to 1 synaptic delay; Eccles, 1964). This agrees well with the average latency (0·5 msec) of the recurrent depression of Ia IPSPs. The maximal effects are in both cases evoked at conditioning–testing intervals below 20 msec and the duration of the recurrent depression is within the same time range.

The effects of the submaximal Ia volleys (the submaximal Ia IPSPs, as well as excitation of interneurones by near threshold stimulation) can be

completely obliterated by volleys in motor axon collaterals. The depression of maximal Ia IPSPs was only partial and may be related to the fact that generation of the spikes evoked in interneurones by maximal Ia volleys was prevented in only 60 % of the cells. In the remaining 40 % there was a decrease in their firing index and an increase in latency of spike generation which may be correlated with the observed increase in latency and time to peak of the Ia IPSPs during recurrent inhibition.

The Ia IPSPs and the monosynaptic excitation of interneurones, evoked from the same Ia afferents, were depressed by antidromic volleys in the same ventral roots (L5 and L6 in the case of effects from Q; L7 and S1 in the case of PBSt). Finally both the depression of Ia IPSPs and the inhibition of interneurones were evoked from α-fibres.

The above discussion leads to the conclusion that *the Ia interneurones inhibited by volleys in recurrent motor axon collaterals in principle have all the known properties required of interneurones mediating reciprocal Ia inhibition to motoneurones.*

Properties of Ia interneurones in the intermediate region. The monosynaptic excitation (I) by volleys in low threshold Ia afferents (II) and capability to follow high frequencies of peripheral stimulation (III) characterize also the Ia interneurones located in the intermediate region (Eccles *et al.* 1956, 1960). It was reported originally (Eccles *et al.* 1956) that they are activated by group Ia afferents from only one group of synergic muscles (IV) and are excited by cutaneous afferents and group III afferents from a variety of muscles (V). Further studies with intracellular recording revealed that in some Ia intermediate interneurones EPSPs are evoked also from Ib afferents from the same or other nerves, as well as from Ia afferents from the nerves which may not be closely related in function (Eccles *et al.* 1960; Hongo *et al.* 1966).

Descending excitatory effects (VI) were found from pyramidal (Lundberg, Norrsell & Voorhoeve, 1962) and rubrospinal tracts (Hongo, Jankowska & Lundberg, 1965; Kostyuk & Pilyavsky, 1969) but monosynaptic excitatory action from the vestibulospinal tract was not observed (S. Grillner, T. Hongo & S. Lund, personal communication). Finally no inhibition (VII) could be detected by volleys in recurrent motor axon collaterals.

The main difference between the Ia interneurones in the ventral horn and in the intermediate region is thus that the latter receive neither recurrent inhibition nor monosynaptic excitation from the vestibulospinal tract.

The question is now whether there may exist two parallel Ia inhibitory pathways to motoneurones, one with and another without recurrent inhibitory control by volleys in motor axon collaterals. The finding that submaximal Ia IPSPs can be completely abolished by volleys in motor

axon collaterals (Hultborn *et al.* 1971*a*, *b*) shows that they are mediated exclusively by interneurones inhibited by antidromic volleys. The partial depression of the maximal Ia IPSPs could be compatible with the existence of two parallel pathways, if the interneurones not inhibited from motor axon collaterals required more summation for their activation. There is, however, no experimental evidence suggesting marked difference in synaptic linkage from Ia afferents to the intermediate and ventral Ia interneurones. Furthermore the partial depression of the maximal Ia IPSPs is satisfactorily explained by the observation that recurrent inhibition does not prevent maximal Ia volley from activating some of the ventral interneurones. *We thus favour the hypothesis that there is only one pathway for reciprocal Ia inhibition to motoneurones and that this inhibition is mediated by the Ia activated interneurones in the ventral horn.*

Morphological consequences. The present investigation leads to some conclusions concerning morphological properties of the interneurones investigated and of the Renshaw cells.

Most of the Ia interneurones inhibited by volleys in motor axon collaterals were found in the spinal segments in which motoneurones with the same peripheral monosynaptic input are located and in which the corresponding afferents enter. Thus if these interneurones mediate reciprocal inhibition to motoneurones 1, 2 or 3 segments away, they must have axons travelling longitudinally for some distance. The opposite arrangement has been postulated for Ia interneurones in the intermediate region. Eccles *et al.* (1956) found Q interneurones at the same segmental levels as the PB and St motor nuclei, as caudally as lower L7. It was concluded that they have short axons and are excited by collaterals of Ia afferents travelling longitudinally from their entry level to lower L7.

Renshaw cells were supposed to be Golgi II cells (Scheibel & Scheibel, 1966) with short axons not extending beyond one segment and branching at the level of location of their somas. The reason for this supposition was that the recurrent inhibition was believed to be confined to one segment (Eccles, Fatt & Koketsu, 1954). However, the anatomists failed to find such cells (for reference, see Scheibel & Scheibel, 1964, 1966) at locations determined in physiological experiments (Eccles, Fatt & Koketsu, 1954; Thomas & Wilson, 1965). Our finding that Ia interneurones in the ventral horn as well as some motoneurones (Hultborn *et al.* 1971*b*) may be inhibited by antidromic volleys in three ventral roots requires that Renshaw cells either have axons projecting to more than one segment or else that they are excited by motor axon collaterals reaching them from some distance. The former is much more probable since Scheibel & Scheibel (1966) showed that the motor axon collaterals terminate within one half segment of the parent soma and according to Ryall (1970) and Ryall & Piercy (1970)

Renshaw cells are excited only from axons leaving the spinal cord close to the level of their location. The suggestion that Renshaw cells may be 'small funicular cells with relatively short axons' (Szentágothai, 1967, cf. also Scheibel & Scheibel, 1966) finds its support in the present investigation.

The authors wish to express their sincere gratitude to Professor Anders Lundberg for many inspiring discussions and for his invaluable help throughout the investigation and in the preparation of the manuscript. Our thanks are also due to Dr Simon Miller for scrutinizing the English and to Mrs Rauni Larsson for excellent technical assistance. This work was supported by the Swedish Medical Research Council (Project No. B70-14X-94-06B).

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J. Physiol. (1971), **215**, *pp.* 637–664
With 8 text-figures
Printed in Great Britain

RELATIVE CONTRIBUTION FROM DIFFERENT NERVES TO RECURRENT DEPRESSION OF Ia IPSPs IN MOTONEURONES

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(*Received 20 July 1970*)

SUMMARY

1. The pattern of depression of Ia IPSPs by volleys in recurrent motor axon collaterals was investigated in motoneurones supplying hind-limb muscles in the cat. The test IPSPs were evoked by stimulation of dorsal roots and the conditioning antidromic volleys by stimulation of motor fibres in different peripheral muscle nerves.

2. In all motor nuclei investigated the strongest depression of Ia IPSPs is evoked from motor fibres to muscles whose Ia afferents produce the IPSPs. For example, the Ia IPSP from the knee extensor recorded in motoneurones to a knee flexor is most effectively depressed by antidromic stimulation of motor fibres to the knee extensor.

3. The origin of recurrent inhibition of α-motoneurones and of Ia inhibitory interneurones with the same Ia input display a striking similarity. This suggests that the same population of Renshaw cells mediates effects to motoneurones and to Ia inhibitory interneurones.

4. The functional significance of impulses in motor axon collaterals was discussed and it was suggested that they have an important role in the control of the excitatory as well as inhibitory Ia actions to motoneurones. The recurrent inhibition may limit the Ia effects to excitation of homonymous motoneurones, which would provide optimal conditions for control of individual muscles via the γ-loop.

INTRODUCTION

The experiments described in the preceding papers (Hultborn, Jankowska & Lindström, 1971*a*, *b*) revealed that antidromic stimulation of the ventral roots depresses transmission in the disynaptic inhibitory pathway from primary endings of muscle spindles (group Ia afferents) to moto-

neurones. The aim of the present study was to analyse what might be the functional significance of this recurrent inhibitory control by investigating which motoneurone axon collaterals evoke depression of transmission in Ia inhibitory pathways to different species of motoneurones. Peripheral nerves (instead of ventral roots) have therefore been used for conditioning stimulation. The pattern of contribution from different nerves to the recurrent depression of Ia IPSPs has been further compared with the pattern of Ia IPSPs (R. M. Eccles & Lundberg, 1958) and of recurrent inhibition (Renshaw, 1941; Eccles, Fatt & Koketsu, 1954; Wilson, Talbot & Diecke, 1960; Eccles, Eccles, Iggo & Ito, 1961) evoked in motoneurones from the same nerves. A short preliminary report of some of the results has been published (Hultborn, Jankowska & Lindström, 1968).

METHODS

Preparation. The experiments were performed on eleven cats anaemically decorticated (Andén, Jukes, Lundberg & Vyklický, 1966). Most cats were, in addition, maintained under light anaesthesia with pentobarbitone sodium (Nembutal, Abbot, *ca.* 25 mg/kg). Essentially the same results were obtained in the anaesthetized and unanaesthetized preparations, with the exception that the recurrent facilitation (disinhibition) of motoneurones was abolished by Nembutal (Wilson & Talbot, 1960; Hultborn *et al.* 1971*a*). Three cats were spinalized at low thoracic level. No systematic differences were recognized between the spinalized and the non-spinalized preparations.

A schematic drawing of the experimental arrangement is shown in Fig. 1. Intracellular recording was made from antidromically identified α-motoneurones in L5-S1 spinal segments. The L4-S2 dorsal roots (DRs) were transected and proximal parts of the L5-S1 DRs were mounted for stimulation. The L6 and L7 DRs were usually divided into two halves, rostral and caudal, and stimulated separately. The segmental levels at which afferents from different nerves reached the spinal cord were determined by recording incoming afferent volleys from the distal parts of the cut dorsal roots. Test IPSPs were evoked by stimulation of afferent fibres within the cut dorsal roots. The motor axons were activated by stimulation of different muscle nerves of the ipsilateral hind limb. Conditioning stimulation of the nerves was maximal or supramaximal for α-fibres. The conditioning–test intervals varied between 8 and 20 msec. Within these limits the strongest depression of IPSPs is evoked (Hultborn *et al.* 1971*a*). The following nerves were dissected and mounted for stimulation, the abbreviations given in parentheses: quadriceps (Q), or vasto-crureus (VCr) and rectus femoris (RF) separately, sartorius (Sart), gracilis (Grac) adductor femoris and longus (Add), posterior biceps-semitendinosus (PBSt) or posterior biceps (PB) and semitendinosus (St) separately, anterior biceps (AB), semimembranosus (Sm), gastrocnemius-soleus (G-S), flexor digitorum and hallucis longus (FDL), plantaris (Pl), and the whole peroneal nerve (Per). Otherwise the dissection, the maintenance of the preparation and recording technique were as described previously (Hultborn *et al.* 1971*a*). The following other abbreviations are used: ventral root (VR) dorsal root (DR), post-synaptic potential (PSP), excitatory post-synaptic potential (EPSP), inhibitory post-synaptic potential (IPSP), recurrent inhibitory post-synaptic potential (RIPSP), flexor reflex afferents (FRA).

Disynaptic Ia IPSPs evoked in motoneurones by stimulation of dorsal roots

In order to obtain conditioning effects only from efferents when stimulating the peripheral nerves, the dorsal roots were cut and the test Ia IPSPs were evoked by stimulation of the proximal end of the dorsal root. The afferent fibres from different nerves are mixed within the dorsal roots and the separation between their functional groups by graded electrical stimulation is more difficult than in the peripheral

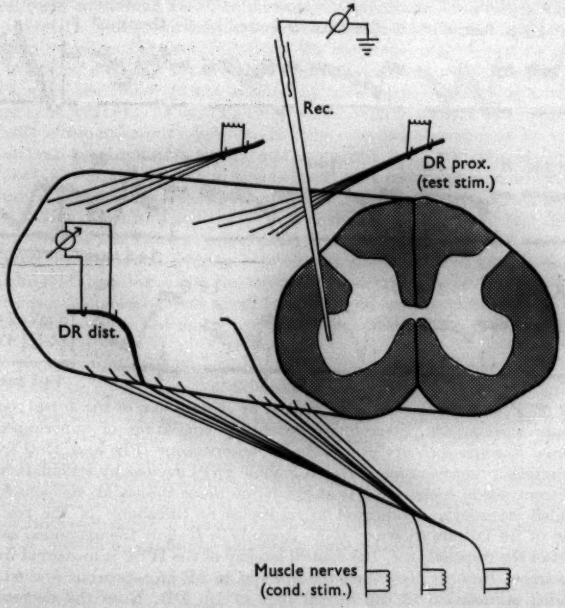


Fig. 1. Schematic representation of the experimental arrangements. See text.

nerves (cf. Lloyd, 1943). However, by selection of dorsal roots used for stimulation and by careful grading of stimulus strengths it was possible to evoke pure or almost pure Ia IPSPs in several groups of motoneurones. In judging the IPSPs as evoked from Ia afferents the following properties of the Ia disynaptic IPSPs were considered:

(i) The Ia IPSPs are evoked by the lowest strengths of electrical stimulation of the nerves (Eccles, Eccles & Lundberg, 1957*a*; Coppin, Jack & McIntyre, 1969).

(ii) The segmental latency of Ia IPSPs is about 1·3 msec (Eccles, Fatt & Landgren, 1956) as compared with about 1·3 or 2·0 msec for di- or trisynaptic Ib IPSPs respectively (Eccles, Eccles & Lundberg, 1957*c*) and 2·0 msec or more for FRA IPSPs (cf. R. M. Eccles & Lundberg, 1959). Also a short duration distinguishes Ia IPSPs markedly from all other IPSPs except those from Ib afferents.

(iii) The Ia IPSPs are evoked with a well-established pattern of distribution,

mainly from the antagonistic muscles (R. M. Eccles & Lundberg, 1958). The disynaptic Ib IPSPs, which may be confused with those from Ia afferents, are evoked mainly in motoneurones supplying extensors (Eccles *et al.* 1957*c*, cf. however, Laporte & Lloyd, 1952).

(iv) The Ia IPSPs are very effectively inhibited by volleys in recurrent motor axon collaterals, while no recurrent inhibition was found of Ib IPSPs and depression of FRA IPSPs, when present, was much weaker (Hultborn *et al.* 1971*a*).

The test IPSPs were evoked by stimulation of the dorsal roots, which contained the highest proportions of group Ia afferents from antagonists. They were accepted

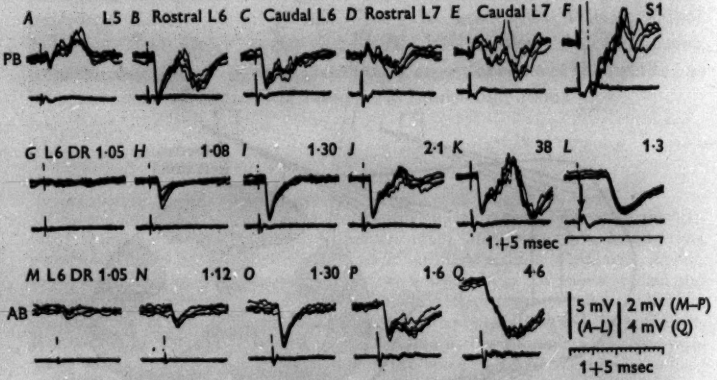

A L5 B Rostral L6 C Caudal L6 D Rostral L7 E Caudal L7 F S1
PB
G L6 DR 1·05 H 1·08 I 1·30 J 2·1 K 38 L 1·3
1+5 msec
M L6 DR 1·05 N 1·12 O 1·30 P 1·6 Q 4·6
AB
5 mV (A–L) 2 mV (M–P) 4 mV (Q)
1+5 msec

Fig. 2. Post-synaptic potentials evoked by stimulation of the dorsal roots. Upper records are intracellular potentials consisting of superimposed traces. Records *A–L* are from a flexor motoneurone (PB) and *M–Q* from an extensor motoneurone (AB). *A–F* show PSPs evoked by stimulation of different dorsal roots (L5–S1) at about ten times threshold. Responses to graded stimulation (indicated in multiples of threshold) of the rostral part of L6 DR are shown in *G–K*. Records in *L* show the expanded early part of the response in *I*. The central latency of the IPSP is measured from the arrow. Records *M–Q* show the PSPs in an AB motoneurone evoked by graded stimulation of the rostral part of L6 DR. Note the decreased amplification in *Q*. Voltage calibrations and time calibration for *A–K* and *M–Q* are to the right of *Q*. Time calibration for *L* is in *L*. In this and the following figures the positivity in intracellular recording and negativity of surface recording are signalled upwards.

as Ia IPSPs if they were evoked at about threshold strength of stimulation, had a latency of about 1·3 msec, were of short duration and could be decreased by conditioning stimulation of at least one of the peripheral nerves. The records *A–F* in Fig. 2 show typical post-synaptic potentials evoked in a knee flexor motoneurone, PB, located in S1 by stimulation of different dorsal roots. In this animal the afferents from the antagonist, the knee extensor Q, reached the spinal cord via both parts of the L6 DRs and the L5 DR. The large short latency IPSPs were evoked from the rostral and caudal parts of L6 DR and a small short latency IPSP from L5 DR. The rostral and caudal parts of L7 DR gave small EPSPs, while S1 DR evoked a strong

homonymous EPSP which easily elicited an orthodromic spike. Records G–K show the responses to graded stimulation of the rostral part of L6 DR. The short latency IPSP appeared at near threshold stimulus strength and was maximal at 1·3 times threshold. This maximal IPSP is shown in L with a faster sweep. Its latency measured from the incoming volley (arrow) is 1·4 msec. Fig. 2, *M–Q*, shows PSPs in a hip extensor motoneurone, AB, evoked by a graded stimulation of the rostral part of L6 DR. The short latency IPSP was maximal at 1·3 times threshold and easily distinguished from later IPSPs evoked by stronger stimuli.

Distinct short latency low threshold IPSPs as in Fig. 2 were evoked only from the dorsal roots containing Ia afferents from antagonists which strongly suggested that they were Ia IPSPs. Furthermore, since the main aim of the investigation was to find from which motor axon collaterals the recurrent inhibition of Ia IPSPs is evoked, it was not critical if the test IPSPs were contaminated with small disynaptic Ib IPSPs, since the latter are not affected by antidromic conditioning stimulation (Hultborn *et al.* 1971*a*). The ensuing error would only be a slight underestimate of the intensity of the recurrent inhibition. In some motoneurones stimulation of dorsal roots gave both monosynaptic EPSPs and disynaptic Ia IPSPs. The purest IPSPs were evoked in PB and St and in some Q and Sart motoneurones, which are all located far from the entry level of the afferents from antagonists. Motoneurones to Sm and AB were difficult to analyse because of the small size of their test IPSPs. In only a minority of Per motoneurones could acceptable test IPSPs be evoked, the reason being the segmental overlap between the entry levels of Ia afferents from Per and those of its antagonists. No acceptable test IPSPs could be evoked in G-S motoneurones which receive only small Ia IPSPs and are also unfavourably located in the same segments as the motor nuclei to their antagonists.

Evaluation of the effectiveness of antidromic stimulation of different nerves

Twenty unconditioned and twenty conditioned test responses were alternatively fed into two halves of the memory of an averaging computer (CAT 1000). The averaged records were then displayed on the oscilloscope and photographed. The effectiveness of the recurrent actions from different nerves has been expressed either as the percentage depression of the test IPSP ('absolute' values) or by scaling the depression from each nerve to the depression from the most effective nerve ('relative' values). The 'absolute' values for depression were calculated by

$$\frac{\text{unconditioned test} - \text{conditioned test}}{\text{unconditioned test}} \times 100,$$

where 0 % means that the test response is unaffected and 100 % that it is abolished by the conditioning stimulation.

The 'relative' depression of the IPSPs indicates the relationship between the effects from each nerve and the most effective nerve:

$$\frac{\text{depression in \% from nerve tested}}{\text{depression in \% from most effective nerve tested}} \times 100,$$

where 100 corresponds to *maximal* conditioning effect, irrespective of how large the 'absolute' depression may be, and 0 implies no effect.

It may be misleading to calculate the means of the 'absolute' values from different individual motoneurones since the depression depends on the strength of the test stimulation and also on the conditioning–test interval (Hultborn *et al.* 1971*a*). These errors are avoided by the use of 'relative' values, although there is also a disadvantage

with this method. The variability of the recorded responses and errors in measurement may reach 5–10 % (in 'absolute' values). In the case of a maximal depression of 40 % a 10 % error will be expressed in 'relative' values as ([10/40] × 100), i.e. 25, while with a maximal depression of 80 % a 10 % error would be only 12·5. To minimize the effects of possible inaccuracies the pattern analysis has been confined to cells showing a depression of at least 50 % from the most effective nerve.

For the same reasons a depression of the test IPSP by 10 % (in 'absolute' values) has not been considered significant. A decrease in the range 11–40 %, 41–70 % and 71–100 % has been arbitrarily defined as weak, strong and very strong respectively.

When conditioning antidromic stimulation produced RIPSPs in the motoneurones sampled it was difficult to prove that the depression of the test IPSP was caused by inhibition of interneuronal transmission (Hultborn *et al.* 1971*a*). Qualitatively, however, it was often possible to decide if the conditioning stimulation evoking RIPSPs in motoneurones also inhibited transmission in the Ia inhibitory pathway. The latter effect was indicated by a more profound decrease of the test IPSP from some nerves than from others, which evoked RIPSPs of the same or higher amplitude.

RESULTS

Contribution from different nerves to the depression of the Ia IPSPs has been analysed in motoneurones to flexors and extensors at different joints. The motor nuclei were selected in order to have representative functional populations in which the patterns of effects on Ia IPSPs could be compared with the known afferent input to the motoneurones and with the pattern of recurrent inhibition to them. The effects from different nerves on test IPSPs in these motor nuclei are presented in Tables 1 and 2. In Table 1 the depression of the IPSPs is expressed in 'relative' values (cf. Methods). In Table 2 these 'relative' values are compared with the percentage depression of the IPSPs in 'absolute' values. Thick and thin hatching correspond to 41–100 and 11–40 % depression respectively. The right-hand column gives percentage decrease of the strongest depression of IPSPs in each species of motoneurones.

Flexors

The hip flexors are represented by Sart, the knee flexors by St and PB and the ankle flexors by all muscles supplied by the common peroneal nerve, Per.

Sartorius. The double-joint muscle Sart consists of two parts, medial and lateral, both working mainly as hip flexors but with different actions at the knee joint. The medial band inserts on the medial side of the tibia and acts as a knee flexor, while the lateral band inserts on the patella and acts as a knee extensor (Sherrington, 1910). In both types of Sart motoneurones Ia IPSPs are evoked from the hip extensors Add, Sm and AB and the knee extensor VCr. In addition motoneurones supplying the medial part receive Ia IPSPs from the double-joint muscle RF (knee

TABLE 1. Contribution from different nerves to the recurrent depression of Ia IPSPs in different motoneurones. The species of motoneurone investigated are listed in the first column to the left. The figures in the second column indicate the numbers of motoneurones investigated. The nerves used for conditioning stimulation are listed above. The resulting depression of Ia IPSPs recorded in each species of motoneurone is presented in the corresponding horizontal rows. The values given are the means of the decrease evoked from different nerves in respect to the maximal effects, i.e. 'relative' values (cf. Methods). The figures in parentheses below the 'relative' values show from how many motoneurones the mean values were calculated, indicating at the same time in how many cells the combination was tested without evoking RIPSPs in the motoneurones recorded from. The large brackets in the column of St and PB indicate when the two nerves were used conjointly for conditioning

Flexors

		Antidromic conditioning nerve volleys											
							St	PB					
Motoneurone	No.	Sart	Add	Sm	AB	Grac			Q	Per	G-S	Pl	FDL
Sart	18	—	100	90	50	37	12		61	5	11	3	4
			(18)	(18)	(16)	(10)	(16)		(9)	(16)	(17)	(17)	(18)
St	19	19	40	22	1	−3	—	5	100	6	0	0	5
		(19)	(19)	(7)	(4)	(13)		(2)	(19)	(5)	(5)	(4)	(7)
PB	13	15	38	23	8	3	−2	—	100	1	2	6	7
		(13)	(11)	(8)	(6)	(10)	(4)		(13)	(7)	(3)	(7)	(7)
Per	14	1	11	19	78	−3	0	19	5	—	100	57	53
		(9)	(11)	(6)	(14)	(9)	(2)	(4)	(12)		(14)	(6)	(11)

Extensors

		Sart	Add	Sm	AB	Grac	St	PB	Q	Per	G-S	Pl	FDL
Sm	15	39	28	—	2	28	—	0	100	0	12	0	2
		(15)	(3)		(3)	(8)		(3)	(15)	(1)	(3)	(1)	(2)
AB	10	25	48	22	—	13	2	2	100	6	10	6	7
		(10)	(5)	(3)		(10)	(2)	(3)	(10)	(7)	(1)	(3)	(7)
Q	23	10	16	78	85	24	100		—	49	48	28	35
		(8)	(8)	(13)	(21)	(17)	(23)			(19)	(20)	(10)	(14)

extensor and hip flexor) (R. M. Eccles & Lundberg, 1958). The test IPSPs used for pattern analysis were usually evoked from the caudal part of L 6 DR or L 7 DR because there is no contamination of monosynaptic EPSPs from these roots. The IPSPs from the caudal part of L 6 DR were evoked from Add, Sm and to a small extent Q afferents, while those from L 7 DR were evoked mainly from AB and Sm.

TABLE 2. Comparison of the contribution from different nerves to the recurrent depression of Ia IPSPs in different species of motoneurone expressed in 'relative' and 'absolute' values. The motor nuclei investigated are listed to the left, while the nerves used for antidromic stimulation are indicated above. The figures in the Table are the means of the 'relative' decrease of the IPSPs and are the same as in Table 1. The decrease expressed in 'absolute' values has been evaluated as strong and very strong (decrease by 41–100%, thick hatching), weak (decrease by 11–40%, thin hatching) and not significant (decrease by 0–10%, no hatching). The mean values of the 'absolute' decrease evoked by the most effective nerve in each motoneuronal group are given in the right hand column

Nerves stimulated

Motoneurones	Sart	Add	Sm	AB	Grso	St	PB	Q	Per	G-S	Pl	FDL	max. decrease in %
Sart	—	100	90	50	37	12		61	5	11	3	4	85
Sm	39	28	—	2	28	—	0	100	0	12	0	2	75
AB	25	48	22	—	13	2		100	6	10	6	7	73
St	19	40	22	1	−3	—	5	100	6	0	0	5	95
PB	15	38	23	8	3	−2	—	100	1	2	6	7	88
Q	10	16	78	85	24	100		—	49	48	28	35	72
Per	1	11	19	78	−3	0	19	5	—	100	57	53	80

Decrease by 0–10 % (in 'absolute' values) not significant

Decrease by 11–40 % (in 'absolute' values) weak

Decrease by 41–100 % (in 'absolute' values) strong and very strong

In the Sart motoneurones illustrated in Fig. 3 the test Ia IPSP was evoked from the rostral part of L 6 DR. Records *C–N* show the effects of conditioning antidromic volleys in different peripheral nerves. The test was almost completely abolished from the hip extensors Add and Sm and considerably decreased from AB. As the recurrent effects on Ia IPSPs are

very selective (Hultborn *et al.* 1971*a*) the strong depression of the test IPSP shows that the contribution from other than Ia afferents was negligible. Volleys in the knee extensor Q (note also the recurrent inhibition of the motoneurones in records *D* and *H*) and the knee flexor Grac evoked a smaller decrease, while PBSt and ankle flexors as well as extensors

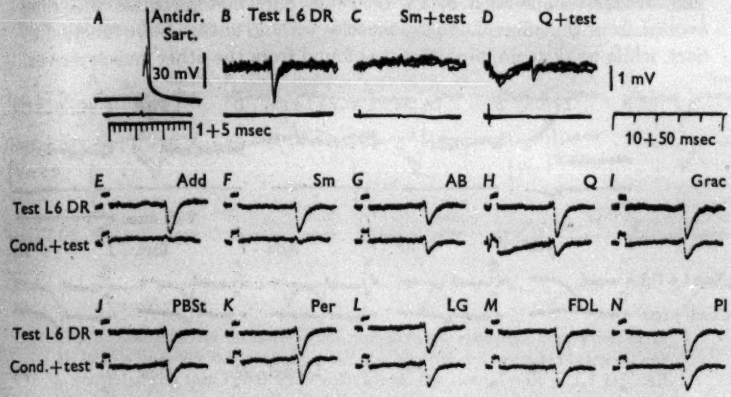


Fig. 3. Contribution from different nerves to the recurrent depression of Ia IPSPs in a Sart motoneurone. In *A–D* the upper traces are intracellular potentials and the lower traces are cord surface potentials. *A* shows the antidromic spike potential from Sart. The test IPSP (*B*) evoked from the rostral part of L6 DR was greatly reduced by preceding stimulation of Sm (*C*) and Q (*D*). *E–N* are the averaged intracellular potentials of twenty test (upper records) and twenty conditioned (lower records) alternated responses. The nerves used for conditioning stimulation are indicated above the upper traces. The conditioning–testing interval was the same for the whole series as in *C* and *D*. The records in this and in the following Figures are presented in the order which corresponds approximately to the effectiveness of the conditioning stimulation in depressing the test IPSPs. The differences in amplitudes of the test IPSPs were probably the result of fluctuations of the membrane potential. However, the percentage decrease was never significantly changed on later conditioning stimulation with the same nerve. Time and voltage calibrations for the intracellular records in *A* are in *A* and for *B–D* to the right of *D*. The calibration pulses at the beginning of the averaged records have an amplitude of 0·5 mV and a duration of 2·0 msec.

had none or only a very weak effect. A similar pattern of effects has been found in other Sart motoneurones. Table 1 shows that the most effective inhibition was from Add (100 in 'relative' values) and that volleys in *Q* also had an effect on the test IPSP without giving a recurrent inhibition of the motoneurones themselves. The small effects from Per, Pl and FDL

indicated in Table 2 were below the significance level, while those from PBSt and G–S were just liminal. The effects from Sart itself could be analysed only in a few units in which the spike was blocked, or was elicited only with the highest stimulus strength within the α-range and in which the autogenous RIPSP was small. No decrease of the test IPSP was then seen. The strongest depression of Ia IPSPs in Sart motoneurones was thus evoked from the efferents to the muscles with an antagonistic function to Sart, while weak or no inhibition was found from the other muscle nerves.

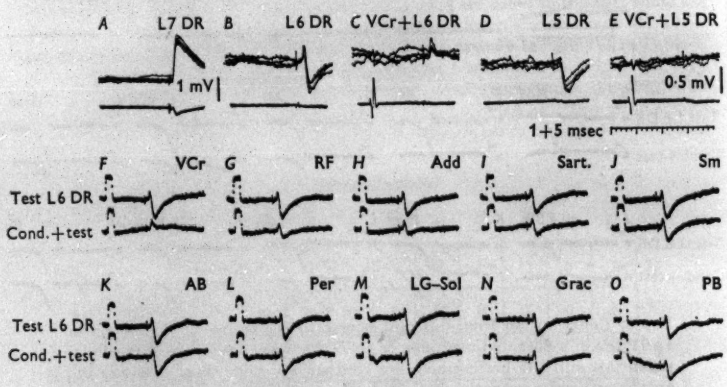


Fig. 4. Contribution from different nerves to the recurrent depression of IPSPs in an St motoneurone. In *A–E* the upper traces are intracellular potentials and the lower traces are cord surface potentials. In *F–O* all the traces are the averaged intracellular potentials. Records in *A*, *B* and *D* show short-latency PSPs evoked by stimulation of the caudal part of L7 DR, of the rostral part of L6 DR and of the L5 DR, respectively. In *C* and *E* the stimulation of the L6 and L5 DRs was preceded by stimulation of VCr. *F–O* show effects of conditioning antidromic volleys in different nerves on a test IPSP evoked from the rostral part of L6 DR. Voltage calibration in *A* is for *A*, and in *E* for *B–E*. Time calibration in *E* is for *A–E*. Other indications are as in Fig. 3.

St and PB. In these knee flexors the Ia IPSPs are evoked from VCr, RF and the lateral part of sartorius (from the latter only in St motoneurones) (R. M. Eccles & Lundberg, 1958). Fig. 4 shows intracellular records from a St motoneurone. In *B* the test is evoked from the rostral part of L6 DR which contained afferents from the VCr, RF and Grac nerves. The IPSP is preceded by a small monosynaptic EPSP, probably due to volleys in Grac afferents which give heteronymous monosynaptic EPSP in St motoneurones (Eccles, Eccles & Lundberg, 1957*b*). Conditioning stimulation of VCr virtually abolished the IPSP leaving the

EPSP unaffected (*C*). L5 DR contained afferents from Sart, VCr and RF but not from Grac and only the disynaptic IPSP (*D*) was evoked by weak stimulation of this root. This IPSP was also completely abolished from VCr. A considerable decrease of Ia IPSPs was also evoked from RF and Add. Smaller effects were seen from Sart and Sm. The means calculated for the whole group of St motoneurones (Table 1) show that conditioning stimulation of Q regularly evoked the strongest decrease. The 'absolute'

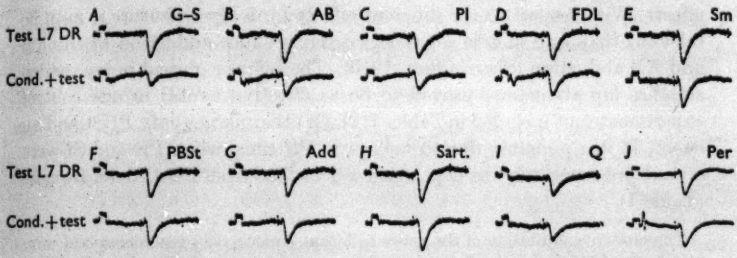


Fig. 5. Contribution from different nerves to the recurrent depression of Ia Ia IPSPs in a Per motoneurone. All the records are averaged intracellular potentials. The test IPSP was evoked from the caudal part of L7 DR. All the indications are as in Fig. 3.

decrease was 95% and thus close to a complete depression of the IPSPs. Of the other nerves tested only Add, Sm and Sart had significant effects (Table 2). Effects from PB could be evaluated quantitatively only in two cells because antidromic stimulation of this nerve usually evoked RIPSPs. Our conclusion that there is no depression from PB is, however, supported by records such as Fig. 4 O, showing hardly any decrease after stimulation of PB in spite of the RIPSP. Similarly no decrease of the test was produced by volleys in the St nerve when the autogenous RIPSPs were lacking or small. Tables 1 and 2 show that the transmission in Ia inhibitory pathways to PB and to St motoneurones was inhibited from the same nerves. In both these groups of motoneurones the strongest depression of IPSPs was evoked by volleys in efferents to antagonistic muscles, but stimulation of nerves to some other extensors (Add, Sm) of seemingly unrelated function also gave some effects.

Per. In ankle and toe flexors innervated by Per the reciprocal Ia IPSPs are evoked from G-S, Pl and FDL (R. M. Eccles & Lundberg, 1959). Test IPSPs with the least contribution of monosynaptic EPSPs were usually evoked from S 1 DR or from caudal part of L 7 DR in motoneurones located in the rostral part of the Per nuclei.

In one of these motoneurones (Fig. 5) conditioning stimulation of G-S

gave a considerable decrease of the test IPSP despite that the latter was nearly maximal (cf. Methods). A smaller depression was evoked from AB, Pl and FDL, while there were no significant effects from other nerves. Tables 1 and 2 show that also in other Per motoneurones the strongest depression of the test IPSPs (mean decrease by 80 %) is produced by volleys in G-S. A strong decrease was evoked also from the ankle extensor Pl, the toe extensor FDL and the hip extensor AB; the latter was nearly as effective as G-S. The other hip extensors, Add and Sm, had only small effects. With respect to the different effects from hip extensors it may be relevant that Add and to some degree Sm produce adduction at the hip and AB abduction (Sherrington, 1910). The inferior gluteal nerve, which supplies hip abductors, proved to be as effective as AB in one control experiment (not included in Tables 1, 2). Of other nerves only PBSt had an effect. It was probably due to volleys in PB since when PB and St were stimulated separately the depression was evoked from PB and not from St (Table 1).

The anatomical division of the nerve to biceps femoris may not correspond completely to the functional subdivision of the muscle, particularly when there is a middle branch between the two more clearly defined AB and PB branches (R. M. Eccles & Lundberg, 1958, cf. Sherrington, 1910). When there was such a middle branch it was removed during the dissection so that the effects from PB would not be contaminated by those from AB.

Thus in motoneurones to ankle and toe flexors the depression of IPSPs was evoked from ankle and toe extensors and one of the hip extensors (AB). The effects from other nerves were weak or absent.

Extensors

The hip extensors are represented by Sm and AB and the knee extensors by Q. Ankle and toe extensors have not been included in the quantitative analysis since it has proved almost impossible to evoke in them satisfactory IPSPs by stimulation of dorsal roots (see, however, below).

Sm and AB. In hip extensors, Sm and AB, the disynaptic Ia IPSPs are evoked from their antagonists Sart and RF and also from the knee extensor VCr (R. M. Eccles & Lundberg, 1958). The test Ia IPSPs are therefore preferably evoked from the L5 DR or from the rostral part of L6 DR through which the afferents from these nerves enter the spinal cord.

In Sm motoneurones the most effective decrease of the test IPSP was evoked by volleys in Q. Table 1 shows that Sart, Add and Grac also depressed the IPSP, while no significant effects were found from the other nerves tested (cf. Table 1, 2). Effects from some of the nerves could be evaluated only occasionally because of large RIPSPs, which were evoked from them in most of Sm motoneurones. Fig. 6 gives typical examples of

records from an Sm motoneurone in which volleys in all nerves except Q (divided into VCr and RF) and Sart evoked RIPSPs of different amplitudes in addition to the possible effect on the Ia inhibitory pathway. The latter could to some extent be judged by comparing the depression of the test IPSP against RIPSPs of similar or different amplitudes (cf. Methods). For example, the more pronounced decrease of the test IPSP by the conditioning stimulation of Add than of the other nerves, despite the weaker RIPSP evoked from Add (cf. records in *C* with *F–I* in Fig. 6), suggested that volleys in Add depressed the transmission in the Ia inhibitory pathway. These observations supplement the material in Tables 1 and 2.

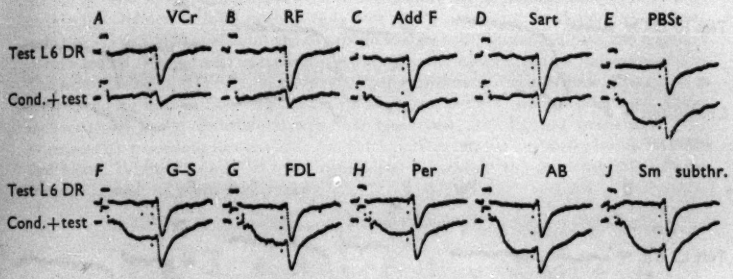


Fig. 6. Contribution from different nerves to the recurrent depression of Ia IPSPs in an Sm motoneurone. All the records are averaged intracellular potentials. The test IPSP was evoked from the rostral part of L6 DR. Stimulation of Sm (*J*) was subthreshold for evoking the antidromic spike potential, though giving recurrent inhibition from lower threshold fibres. All the indications are as in Fig. 3.

A similar pattern has been found in AB motoneurones except for an asymmetry in actions between AB and Sm themselves. The IPSPs in AB motoneurones were decreased by volleys from Sm but not vice versa. In both these hip extensors the Ia IPSPs were inhibited by volleys from the muscles flexing the hip, Sart and RF, although the strongest effects were evoked from Q (both RF and VCr). In addition weaker depression of the test was evoked by conditioning stimulation of Grac, Add and, in AB motoneurones, also Sm.

Q. The Q muscle consists of two functionally different parts, VCr, pure knee extensor and RF, which has a mixed function as knee extensor and hip flexor. In both VCr and RF motoneurones the Ia IPSPs are evoked from knee flexors Grac, PB and St (R. M. Eccles & Lundberg, 1958). The afferents from these muscles reach the spinal cord via L5–S1 DRs, but in order to avoid contamination with large monosynaptic EPSPs from Q afferents in L5–L6 DRs the test Ia IPSP was evoked from S1 or L7 DRs

which contained afferents from St and PB. In most experiments the whole Q nerve was dissected, but in some of them the branches to VCr and to RF were separated in order to permit better identification of motoneurones. Of the twenty-three Q motoneurones in Table 1, three were identified as belonging to VCr and six to RF, but since no systematic differences were recognized all the units are described together. Fig. 7 shows records from a Q motoneurone in which the test IPSP was evoked by stimulation of the L7 DR which contained afferents from PBSt, AB and Sm muscles but not

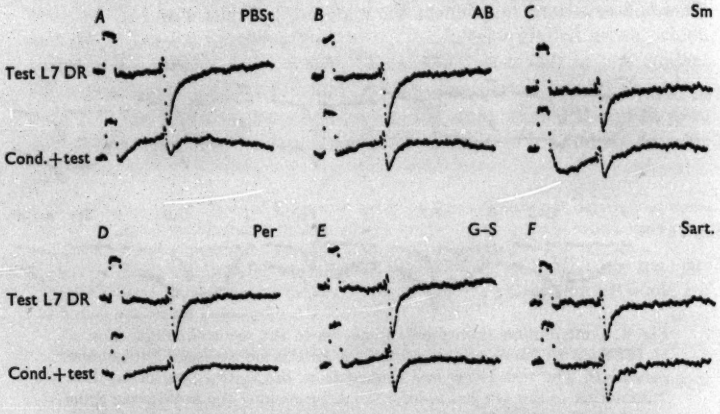


Fig. 7. Contribution from different nerves to the recurrent depression of Ia IPSPs in a Q motoneurone. All the records are averaged intracellular potentials. The test IPSP was evoked from L7 DR. The calibration pulse has an amplitude of 1 mV and duration of 4·0 msec.

from Grac. The Ia IPSP was therefore evoked from PBSt afferents and the small preceding EPSP was most probably from AB and Sm afferents (R. M. Eccles & Lundberg, 1958). The records are representative for the data given in Table 1. They show that the most effective depression of the test IPSPs in Q was evoked from knee flexors PBSt, though volleys from hip extensors AB and Sm could be nearly as effective. Weaker but significant depression of IPSPs (cf. Table 2) was evoked also from Grac, G-S, Pl, FDL and Per, while Sart and Add were without effect.

G-S, Pl, FDL. The antidromic effects on Ia IPSPs in motoneurones belonging to the ankle extensors G-S, Pl and toe extensor FDL have not been included in the quantitative analysis of the material. In most motoneurones the test Ia IPSPs from the dorsal roots were severely contaminated by much stronger monosynaptic EPSPs and other PSPs (cf. p. 641 in

Methods). Acceptable test IPSPs were evoked only in two G-S motoneurones. These IPSPs were decreased by preceding antidromic volleys in the Per nerve.

Supplementary material concerning the origin of RIPSPs in α-motoneurones

One of the aims of the present investigation has been to compare the origin of recurrent inhibition in α-motoneurones with that of the recurrent depression of Ia IPSPs in motoneurones. The distribution of recurrent inhibition from different nerves to α-motoneurones was investigated by Renshaw (1941) and Wilson *et al.* (1960) by antidromic conditioning of monosynaptic test reflexes and by Eccles *et al.*

TABLE 3. The mean amplitudes of RIPSPs generated in lumbar motoneurones by antidromic volleys in hind-limb muscle nerves. The species of motoneurone investigated are listed to the left and the figures in the second column indicate the numbers of cells tested. The muscle nerves used for antidromic stimulation are indicated above. The amplitudes of RIPSPs (in mV) recorded in different motoneurones are given in corresponding horizontal rows. Mean values below 0·1 mV are given as zero. Those motoneurone–nerve combinations which had been investigated previously with intracellular recording are shaded. No values are given for the autogenous RIPSPs (see text). When St and PB were stimulated separately the resulting RIPSPs are shown for each of them separately

Motoneurones	no	Nerves stimulated: Sart	Add	Sm	AB	Grac	St	PB	Q	Per	G-S	Pl	FDL
Sart	30	—	0	0	0	0·2	0		0·7	0	0	0	0
Sm	32	0	0·5	—	1·3	0·1	0·8		0	0·5	0·5	0·5	0·5
AB	32	0	0·4	1·2	—	0	1·4		0·1	0·1	1·5	0·2	0·3
St	26	0	0	0·7	1·0	0·3	—	0·9	0	0·9	0·7	0·3	0·4
PB	20	0	0	0	0·9	0·1	0·7	—	0	0	0·8	0	0
Q	55	0·2	0·8	0·5	0·1	0	0		—	0	0	0	0
Per	80	0	0	0·4	0·1	0	0·3	0	0	—	0·1	0·1	0·3
G-S	8	0	0	0·1	1·5	0	0	0·1	0	0·1	—	0·8	0·8

(1954) and Eccles *et al.* (1961) by means of intracellular recording of RIPSPs. These investigations did not cover all nerve combinations of interest for our comparison. The material collected during the present study is summarized in Table 3 containing the mean amplitudes of the RIPSPs evoked from different nerves except for the autogenous RIPSPs. The autogenous RIPSPs (cf. Methods in Eccles *et al.* 1961) were not calculated systematically but, when measured, they were larger than RIPSPs evoked from other nerves (cf. Eccles *et al.* 1961). Since the analysis of the RIPSPs in motoneurones was not the main aim of the investigation the conditions

under which this supplementary material was collected were not quite uniform and not always optimal. The over-all pattern of RIPSPs is similar to that reported in earlier investigations. Some discrepancies as well as new findings will be mentioned: (i) Sart motoneurones were not previously investigated with intracellular recording. Apart from Sart itself the RIPSPs were also evoked from Q and Grac. Small RIPSPs (mean value below 0·1 mV) were sometimes seen from PBSt. (ii) In Sm motoneurones medium-sized RIPSPs have now been found from Add and Grac (not previously tested). Wilson *et al.* (1960) found a depression of Sm monosynaptic reflex after antidromic conditioning with Q nerve in a majority of cases tested, while Eccles *et al.* (1954) and Eccles *et al.* (1961) failed to observe effects from Q. In our material small RIPSPs from this nerve were seen only rarely (zero in Table 3), but this may be the result of recurrent facilitation frequently evoked in this combination (H. Hultborn, E. Jankowska & S. Lindström, unpublished results). (iii) In AB motoneurones weak RIPSPs were found from Add (not previously tested). (iv) In St motoneurones large RIPSPs from Per were found regularly although Eccles *et al.* (1961) did not find RIPSPs in this combination. Moderate RIPSPs were evoked from Grac (not previously tested). (v) Small RIPSPs from Grac have also been found in PB motoneurones. (vi) Monosynaptic test reflexes recorded in the Grac nerve were considerably decreased after antidromic conditioning stimulation of the St nerve (not included in Table 3). (vii) In Q motoneurones small effects from Sart and Add (not previously tested) have been found. RIPSPs were sometimes evoked also from G-S but the mean value was below 0·1 mV (zero in Table 3). (viii) In Per motoneurones small RIPSPs were sometimes seen from G-S which is in accordance with Eccles *et al.* (1961). In experiments in which the nerve branches to tibialis anterior, extensor digitorum longus and peronei muscles were dissected separately, we did not find any RIPSPs from G-S in motoneurones belonging to tibialis anterior and extensor digitorum longus while motoneurones of the peronei muscles often received them (not shown in Table 3). (ix) Some G-S motoneurones received RIPSPs from Q. Because of a mean below 0·1 mV they are not indicated in the table. Recurrent inhibition was found in this combination by Wilson *et al.* (1960).

DISCUSSION

The recurrent depression of Ia IPSPs will now be related to the recurrent inhibition of motoneurones and to the pattern of Ia inhibitory connexions to motoneurones in order to allow a more general discussion on the functional role of recurrent effects from motor axon collaterals.

Contribution from different nerves to recurrent inhibition of motoneurones

According to Eccles *et al.* (1954) and Eccles *et al.* (1961) the distribution of recurrent inhibition among motoneurones is related mainly to their proximity. From this 'proximity hypothesis' it was postulated that recurrent inhibition has a general suppressor action on motoneurones regardless of their function. Wilson *et al.* (1960) pointed out that the proximity of motor nuclei cannot alone account for the distribution of RIPSPs. A correlation between proximity of motor nuclei and the size of RIPSPs does not in itself exclude connexions depending on functional relations

between muscles since the location of the nuclei is not functionally at random (cf. Romanes, 1951; Thomas & Wilson, 1967). While recurrent inhibition may be present between antagonists in a wider sense, e.g. extensors and flexors (exemplified by the mutual recurrent inhibition between G-S and PB motor nuclei) the most important limitation in the recurrent inhibitory connexions is their *absence between nuclei supplying muscles acting as strict antagonists at the same joint*. This rule is valid also when these motor nuclei have a similar rostrocaudal distribution; the recurrent inhibition is lacking between the motor nuclei of tibialis anterior and G-S (Wilson *et al.* 1960) and in Q motor nucleus from Grac (see Table 3). A suggestion of this rule was given in a paper on recurrent inhibition of forelimb motoneurones (Thomas & Wilson, 1967) and it seems to be established by the present findings on hind-limb nuclei. The weak RIPSPs seen in many Per motoneurones after antidromic stimulation of the nerve to FDL may not be an exception to this rule because most of the Per motoneurones supply muscles acting at the ankle joint (for example tibialis anterior and peronei muscles) which cannot be considered as strict antagonists to FDL which gives plantar flexion of the toes (Sherrington, 1910).

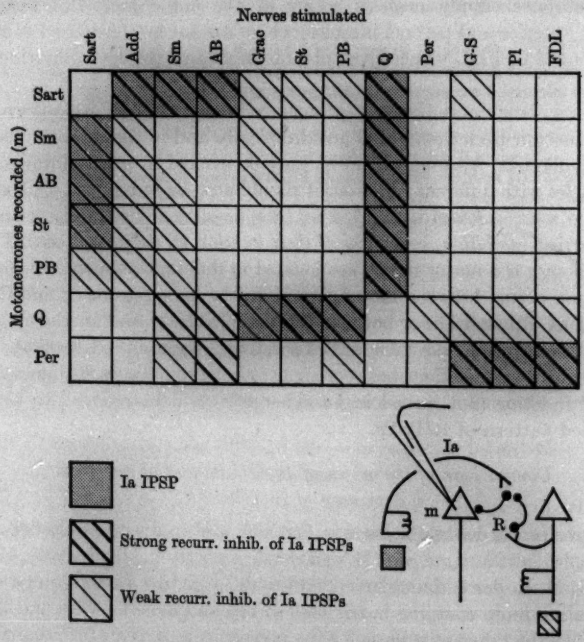
From a given nerve the most pronounced recurrent inhibition is evoked in the motoneurones activated antidromically and in the motor nuclei to close synergists. Although RIPSPs may be evoked in some motor nuclei to muscles with different functions it should now be recognized that *motor nuclei to muscles which are linked in Ia synergism are mutually connected by recurrent inhibition, regardless of their location in the spinal cord*. Thus, even though the motor nuclei are located in different segments recurrent inhibition occurs between motoneurones to the knee flexors, St and Grac, as well as PB and Grac, both in the cat (Table 3) and in the baboon (H. Hultborn, E. Jankowska & S. Lindström, unpublished results). The recurrent interconnexions between motor nuclei to muscles with more unrelated function (not linked in Ia synergism) will be referred to as the extended pattern of RIPSPs.

Comparison of the origin of Ia IPSPs and of recurrent depresson of Ia IPSPs

The reciprocal Ia inhibition was first believed to operate only between antagonists at the same joint (Lloyd, 1946; Laporte & Lloyd, 1952). However, R. M. Eccles & Lundberg (1958) found that the Ia inhibitory connexions are more complex in the case of hip and knee muscles and their results will be taken as a basis for the present comparison. In Table 4 the combinations in which Ia IPSPs are evoked in motoneurones are indicated by dots, while the combinations in which recurrent depression of Ia IPSPs

has been found are hatched. The table shows that the two phenomena overlap to a great extent and that in all cases where the afferent fibres from a muscle nerve evoke Ia IPSPs, the latter are depressed by volleys in the efferents from the same nerve. Recurrent effects are evoked also from some other nerves but the maximal recurrent depression of Ia IPSPs is always evoked from the nerves, whose afferents give Ia inhibition.

TABLE 4. Comparison of the origin of Ia IPSPs and of depression of Ia IPSPs in motoneurones innervating the same muscles. The species of motoneurone investigated are listed to the left and the nerves whose orthodromic and antidromic effects are being compared are indicated above. The diagram shows the stimulating and recording arrangements of the corresponding experiments (m = motoneurones, R = Renshaw cell). The combinations in which the Ia IPSPs are evoked from a given nerve (based on results by R. M. Eccles & Lundberg, 1958) are marked with dots. Hatching indicates those combinations in which the Ia IPSPs are depressed by antidromic stimulation of the nerves. Thick and thin hatching correspond to strong (41–100 %) and weak (11–40 %) recurrent effects, respectively, as in Table 2.



Comparison of the origin of recurrent inhibition of motoneurones and of recurrent depression of Ia IPSPs

As discussed above the recurrent inhibition of motoneurones is to a very large extent drawn from synergists while the depression of Ia IPSPs is mainly evoked from antagonists. Thus there may be a roughly negative correlation between these two recurrent effects. However, Table 5 shows that in a number of combinations the inhibitory effects on motoneurones and the depression of Ia IPSPs did not exclude each other, although when they overlapped either both or one of the effects were weak, the single exception being the effects from Q to Sart motoneurones. This combination needs further analysis since both Q and Sart muscles have a complex function with some portions of them acting as synergists. Furthermore the Ia inhibitory connexion from Q to motoneurones of Sart is associated with the hip flexor function of the Sart muscle (R. M. Eccles & Lundberg, 1958) and represents an exception from the more common pattern of reciprocal Ia inhibition from strict antagonists.

Comparison of the contribution from different nerves to recurrent inhibition of motoneurones and of interneurones mediating Ia inhibition

The interneurones mediating Ia inhibition can be defined both with respect to the Ia afferents supplying their monosynaptic excitation and to the motor nuclei inhibited by them. The approximate degree to which they are inhibited from motor axon collaterals can be deduced from the depression of Ia IPSPs in the motoneurones inhibited by them. For example, the Ia inhibitory interneurones with monosynaptic Ia excitation from the knee flexor St will inhibit knee extensor Q motoneurones. Since the Ia IPSPs in Q motoneurones are strongly decreased after antidromic conditioning volleys in PBSt, Sm and AB and also weakly from Grac, Per, G-S, FDL and Pl it is assumed that stimulation of these nerves inhibits, with a corresponding intensity, the interneurones monosynaptically excited from St. In Table 6 the origin of recurrent effects on interneurones mediating Ia IPSPs is compared with the origin of recurrent inhibition of motoneurones. Effects to motoneurones (m_1) and interneurones (i) with the same main monosynaptic Ia connexions are indicated in the same row, the origin of the Ia afferents listed in the column to the extreme left. The motor nuclei (m_2) inhibited by the interneurones are listed in the second column. There is either one row for one kind of monosynaptic afferent connexions or a few rows if interneurones excited by afferents from one muscle (Sart and Q) are mediating inhibition to different motor nuclei. In the latter cases the origin of recurrent inhibition to these interneurones has

TABLE 5. Comparison of the origin of RIPSPs in motoneurones and of recurrent depression of Ia IPSPs. The species of motoneurone investigated are listed to the left, while the nerves used for antidromic stimulation are indicated above. The diagram shows the stimulating and the recording arrangement (m = motoneurones recorded from, R = Renshaw cells) when investigating the recurrent effects on motoneurones and on transmission in Ia inhibitory pathways. The distribution of RIPSPs is based on the earlier investigations supplemented by the material summarized in Table 3. The recurrent inhibition is arbitrarily divided into three groups, strong (> 0·6 mV, dense dotted), weak (0·1–0·6 mV, sparse dotted) and absent or very weak (< 0·1 mV, without dots). If different values of the RIPSPs were found in the available material the following supplementary criteria were used: (1) the recurrent inhibition was estimated as strong when reported as strong in most studies; (2) it was classified as weak when it was reported as weak or absent in most studies, even if values > 0·6 mV were given in some studies; (3) it was estimated as absent when it was reported as absent in some studies and about 0·1 mV in others. Combinations in which the antidromic conditioning stimulation causes a strong or a weak depression of Ia IPSPs are indicated with thick and thin hatching, respectively, as in Tables 2 and 4

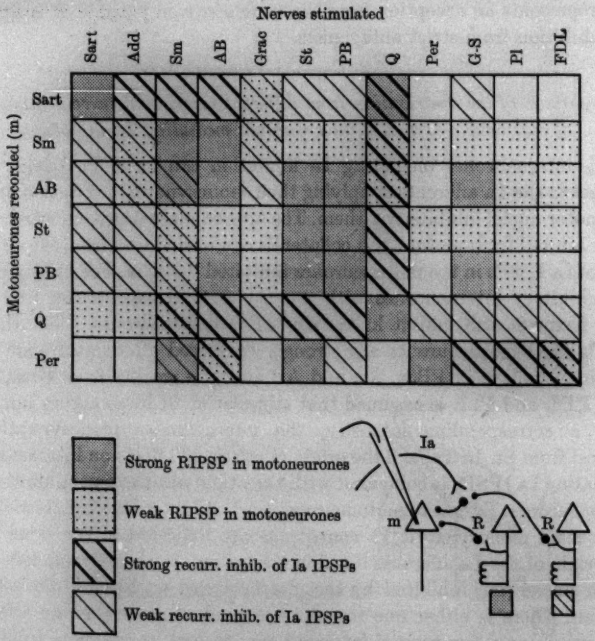
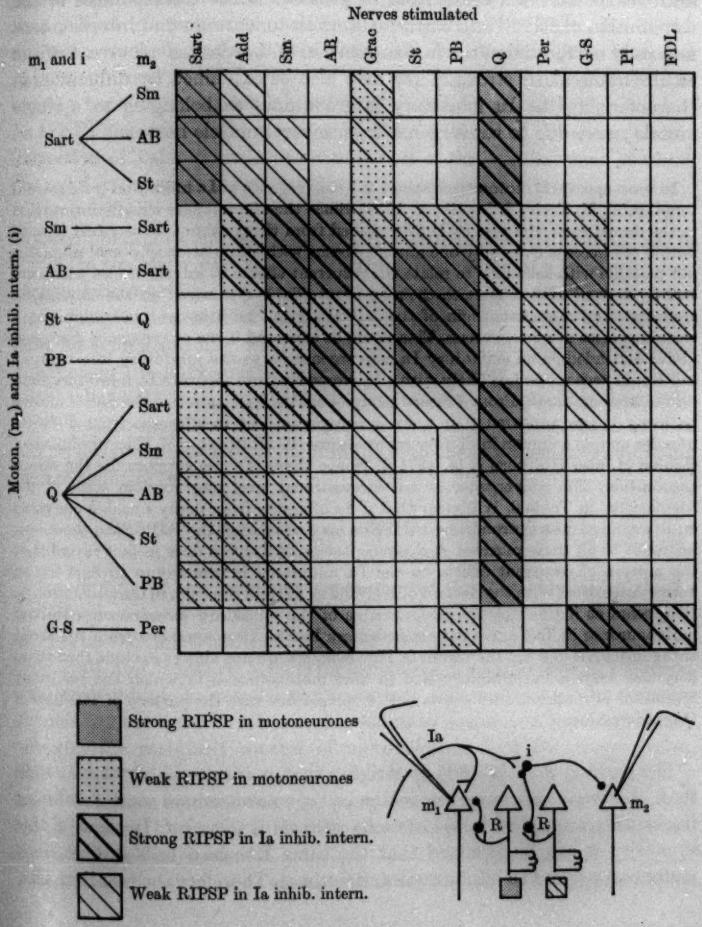


TABLE 6. Comparison of the origin of recurrent inhibition of motoneurones and interneurones mediating Ia inhibition. The origin of Ia afferents with monosynaptic connexions with the motoneurones (m_1) and the interneurones (i) is indicated to the extreme left. In the second column the species of motoneurones (m_2) receiving inhibition from the interneurones (i) are listed. The nerves used for antidromic stimulation are indicated above and their effects on the motoneurones and the interneurones are indicated by dotted and hatched areas respectively. The diagram shows the recording and stimulating arrangements in the different experiments from which the distribution of the recurrent effects were obtained



been deduced separately, from recurrent effects on IPSPs in these different motor nuclei.

Table 6 reveals a marked parallelism between recurrent inhibitory effects to motoneurones and to interneurones receiving the same Ia excitatory input. Large RIPSPs in motoneurones are always matched by inhibition of the corresponding interneurones and the postulated strong recurrent inhibition of interneurones is always (with a single exception) combined with RIPSPs in motoneurones excited by the same afferents. In most cases also weaker RIPSPs are evoked in parallel. Minor discrepancies in the distribution of RIPSPs to corresponding motoneurones and interneurones may only mirror difficulties in classification of 'borderline' recurrent effects as absent, weak or strong. They may also be explained by difficulties in characterizing the Ia inhibitory interneurones as belonging to a single muscle nerve due to convergence from several muscle nerves.

In most species of motoneurone there is a convergence of Ia inhibition from several muscles (R. M. Eccles & Lundberg, 1958). Since the afferents are usually intermixed in the dorsal roots the test Ia IPSPs evoked from them cannot be ascribed to activation of afferents from only one muscle. Hence with stimulation of dorsal roots it is not possible with certainty to relate the recurrent effects to interneurones which are strictly defined with respect to their Ia input. Table 6 is based on the simplifying assumption of a similar origin of the RIPSPs in all Ia interneurones inhibiting a certain species of motoneurone. This would be expected if the convergence discussed above depended on an excitatory Ia convergence on to the inhibitory interneurone but would perhaps be less likely with a convergence from separate Ia inhibitory lines on the motoneurones. From a recent study of spatial facilitation in the Ia inhibitory pathway it has appeared that there is convergence of Ia afferents from different muscles on to a common inhibitory interneurone (H. Hultborn & M. Udo, unpublished results, cf. also Hultborn *et al.* 1971*b*). Hence there is some support for the above assumption. The convergence at an interneuronal level may explain some of the 'deviations' in Table 6. It is clear that if Ia afferents from many muscles converge on to common interneurones their RIPSPs may not match the RIPSPs in the motoneurones to all these muscles. A close inspection of Table 6 does in fact reveal that the pattern of recurrent effects on the Ia inhibitory interneurones to Sart shows better correlation with the pattern of RIPSPs in AB and Sm than in Q motoneurones. It appears as if the RIPSPs receptiveness of the inhibitory interneurones to Sart motoneurones in Table 6 is determined more by the Ia connexions from the strict antagonists AB and Sm than from Q. However, we are not able to exclude that there may also exist a Ia inhibitory line to Sart motoneurones in which the recurrent inhibition of interneurones shows better correlation with the pattern of RIPSPs in Q motoneurones.

The overlap in Table 6 is so striking that it is reasonable to conclude that *the origin of recurrent inhibition of motoneurones and inhibitory interneurones supplied by the same Ia afferents is identical.* In view of this similarity it can be assumed that the same Renshaw cells inhibit both motoneurones and Ia inhibitory interneurones. Therefore the two Renshaw

cells indicated in the small inset figure in Table 6 may be substituted with a common interneurone mediating both effects.

Earlier, we differentiated between the recurrent inhibition among motor nuclei to Ia synergists and among motor nuclei to muscles of more unrelated function (the extended pattern of recurrent inhibition). This extended pattern of recurrent inhibition of motoneurones is also associated with a similar pattern of recurrent inhibition of inhibitory interneurones with the same Ia input (Table 6). Corresponding to the recurrent inhibition of G-S motoneurones from AB there is, for example, recurrent inhibition from AB of interneurones mediating reciprocal inhibition to Per motoneurones, evoked among others from G-S Ia afferents. The extended pattern of recurrent inhibition is also of interest with regard to the overlap between recurrent inhibition of motoneurones and depression of Ia IPSPs discussed in the previous section (cf. Table 5). However, a close inspection of Table 5 reveals that only half of the combinations in which the overlap occurred is associated with the extended pattern.

The principal connexions from motor axon collaterals via Renshaw cells to α-motoneurones and to Ia inhibitory interneurones are summarized in Fig. 8. In the lower part there is a schematic drawing of a joint with two antagonistic muscles, m_1 and m_2. Group Ia muscle afferents from m_1 have monosynaptic connexions with motoneurones innervating this muscle (and its synergists) and with interneurones which inhibit motoneurones innervating the antagonistic muscle (represented by m_2). The recurrent axon collaterals from axons to the m_1 muscle activate Renshaw cells which inhibit motoneurones to this muscle (and its synergists) as well as the Ia interneurones mediating inhibition to the motoneurones supplying the antagonists (m_2).

Functional significance of recurrent effects mediated by motor axon collaterals

The finding of the recurrent depression of the transmission in the Ia inhibitory pathway (Hultborn *et al.* 1971*a*) has revealed important new aspects on the functional significance of recurrent effects from motor axon collaterals and Renshaw cells. The conclusion that impulses in recurrent collaterals inhibit in parallel motoneurones and inhibitory interneurones supplied by the same Ia afferents has led us to postulate a functional unity between the recurrent control of motoneurones and Ia interneurones. Since the latter regulate the transmission of Ia inhibition it seems likely that the former controls the excitatory transmission from Ia afferents to motoneurones. Recurrent inhibition may thus have its special significance in γ-controlled movements.

The wide distribution of excitatory as well as inhibitory Ia connexions

from some muscles has made it difficult to understand the role of the γ-system in the regulation of more precise movements (R. M. Eccles & Lundberg, 1958). The function of the recurrent inhibition may be to limit the extent of the diverse Ia effects. The prevailing pattern of the recurrent depression of Ia IPSPs indicates that during operation of the Renshaw

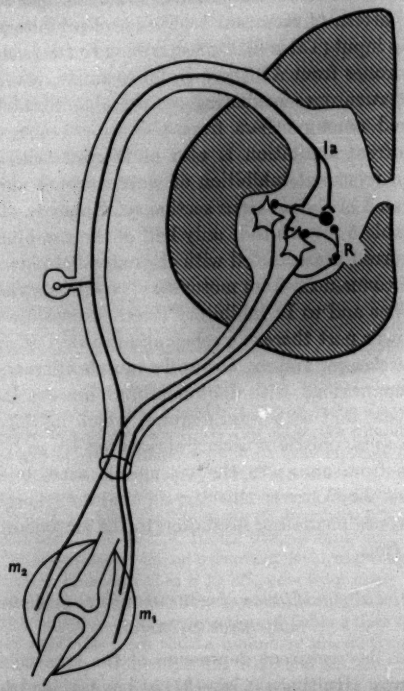


Fig. 8. Diagram summarizing the principal connexions from recurrent motor axon collaterals, via Renshaw cells (R), to motoneurones and to Ia inhibitory interneurones. Filled black circles indicate inhibitory neurones. For further description see text.

loop, activity in one motor nucleus will release motoneurones to antagonists from the Ia inhibitory control. When activity in γ-efferents to a muscle evokes a Ia discharge which gives excitation of α-motoneurones the activity in the Renshaw loop may prevent a concomitant reciprocal inhibition of motoneurones to antagonists. This would already be an important functional restriction of the potential effects evoked by Ia

afferents. It is possible that the recurrent inhibitory effects on α-motoneurones (among motor nuclei linked in Ia synergism) further limit Ia actions by depressing their excitatory effects on motoneurones. A blockage of the recurrent pathways is accompanied by a large increase of heteronymous reflexes (Brooks & Wilson, 1959). The Renshaw inhibition of motoneurones may thus act as a type of lateral inhibition and prevent heteronymous Ia EPSPs from exciting motoneurones. The restriction of the Ia effects to excitation of homonymous motoneurones would thus provide optimal conditions for control of individual muscles via the γ-loop and allow complex movements or postures involving co-contraction of flexors and extensors at the same joint.

The discussion of the dominating pattern of recurrent interconnexions between motor nuclei to Ia synergists disregards the more complex distribution of some of the recurrent effects, referred to above as the extended pattern. Its function is unknown but some indication is given by the effect from AB on G-S motoneurones with the associated depression of Ia IPSPs in Per motoneurones. Since there were no, or only very weak, effects from the other hip extensors, Sm and Add, it seemed possible to associate the effect from AB with its abductor function. This idea is supported by the finding that the inf. gluteal nerve (supplying hip abductors) has similar effects as AB on both G-S motoneurones (cf. Eccles *et al.* 1961) and on Ia inhibitory transmission from ankle extensors to Per motoneurones. This finding suggests that muscles with extended recurrent connexions may be linked in 'functional synergism' in certain movements or postures, which could also explain these connexions in similar terms as between Ia synergic muscles.

Renshaw cells are subject to spinal control (Eccles *et al.* 1954; Frank & Fuortes, 1956; Curtis, Phillis & Watkins, 1961; Wilson, Talbot & Kato, 1964; Curtis & Ryall, 1966) as well as supraspinal control (Koizumi, Ushiyama & Brooks, 1959; Holmqvist & Lundberg, 1959; Granit, Haase & Rutledge, 1960; Haase & van der Meulen, 1961; MacLean & Leffman, 1967), which can either enhance or depress the transmission in the recurrent pathway. The extensive supraspinal control of Renshaw cells indicates that they not only subserve segmental mechanisms but are also one of the elements of the supraspinal control of reflex activity.

In which situations then are the recurrent pathways brought into play and when are they blocked? Severin, Orlovskii & Shik (1968) have recently reported that Renshaw inhibition of motoneurones is considerably depressed during stepping in mesencephalic cats. The Renshaw cells are also profoundly inhibited (Bergmans, Burke & Lundberg, 1969) during activation of neuronal system of particular interest in relation to stepping (Jankowska *et al.* 1967). The experimental evidence suggesting that the

transmission in the recurrent pathways is blocked during stepping is thus well in keeping with the recently postulated importance of the full excitatory and inhibitory Ia patterns in the regulation of the gait (Engberg & Lundberg, 1969; Lundberg, 1969).

Our conclusion is therefore that the operation of the Renshaw loop subserves complex movements or postures involving a co-contraction of antagonistic muscles by restricting the effects of impulses in Ia afferents to excitation of homonymous motoneurones and that the recurrent pathways are depressed during movements like stepping which require the entire Ia reflex pattern. If so, there may be a similar organization of the recurrent pathways in the spinal segments from which the forelimb muscles are innervated. Such an organization might be absent among motoneurones supplying the intercostal muscles and diaphragm, since the movements executed by them are more stereotyped than in the case of hind- and forelimb muscles. Accordingly recurrent actions have been found among motoneurones supplying forelimb muscles (Thomas & Wilson, 1967), but no significant recurrent effects have been found among motoneurones innervating intercostal muscles (Sears, 1964) and among motoneurones to the diaphragm (Gill & Kuno, 1963).

The authors wish to express their sincere gratitude to Professor Anders Lundberg for many inspiring discussions and for his invaluable help throughout the investigation and in the preparation of the manuscript. Our thanks are also due to Dr Simon Miller for scrutinizing the English and to Mrs Rauni Larsson for excellent technical assistance. This work was supported by the Swedish Medical Research Council (Project No. B70-14X-94-06B).

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J. Physiol. (1971), **215**, *pp.* 655–678
With 1 *text-figure*
Printed in Great Britain

GROWTH AND REPRODUCTION OF MICE CROSS-FOSTERED BETWEEN PARENTS REARED AT DIFFERENT TEMPERATURES

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(*Received 6 October 1970*)

SUMMARY

1. Litters of mice, *Mus musculus*, of the highly inbred strain A2G/Tb, were reduced to four at birth and cross-fostered within and between three classes: (i) bred at 21° C (controls); (ii) the first generation reared at −3° C ('new stock'); (iii) the seventeenth to twenty-third generations reared at −3° C ('old stock'). There were therefore nine classes of fostered mice.

2. There was a higher death rate in the nest and after weaning among mice of new-stock parentage, regardless of foster-parentage.

3. Litters reared at 21° C were heavier at 3 weeks than those reared at −3° C, regardless of parentage; the effect of temperature was also evident in body weights at 16 weeks. There was compensatory growth between 3 and 16 weeks, shown by lower variance in body weight, within classes, at 16 weeks.

4. Members of fostered litters were mated, and their reproductive performance recorded to the age of 28 weeks.

5. More young were born and weaned per pair at 21° C than at −3° C, regardless of true parentage. In contrast, nestling mortality depended principally on true parentage; there were fewer deaths among the young of mice whose true parents were old stock, regardless of foster-parentage; this effect was especially evident for losses of whole litters.

6. Mean body weights of the nine classes of fostered females were positively correlated with mean numbers of young born to them, and with the mean weights of their young at 3 weeks; but there was no such correlation with death rates among their young.

7. Young of some of the fostered mice were also mated. The effect of old-stock ancestry on nestling mortality was not evident in this further generation. Some mice were studied after transfer between temperatures without fostering. Their breeding performance confirmed that old-stock mice did not retain their superiority after two generations at 21° C.

8. General conclusions are: (i) differences of growth between classes of

fostered mice reflected the temperature in which the mice were reared, whereas mortality among fostered mice was influenced by the conditions they had experienced *in utero*; (ii) the production of young by the fostered mice was influenced by environmental temperature, while the death rates among these young in the nest reflected the conditions experienced *in utero* by their parents.

INTRODUCTION

Physiological adaptation to cold by mammals is usually studied among laboratory rodents kept alone or in small groups of like sex. When mammals are required to breed in a cold environment they must undergo adaptive changes beyond those displayed in such experiments (Barnett, 1965; Barnett & Widdowson, 1971); the females have to adjust their metabolism and their behaviour to the demands both of the environment and of their young. The environment provided by the uterus and in the nest may then be affected, not only by the conditions in which a female is living during pregnancy and lactation, but also by the character of the environment in which she was reared; and this may be affected in turn by her mother's early environment. Moreover, the development of the young is determined, not only by the environments provided by the mother, but also by the direct action of the inanimate surroundings during and after the nestling phase. In the experiments described below laboratory mice, *M. musculus*, were fostered, within 24 hr of birth, on to females reared in an environment different from that of the true parents. This has made possible a partial analysis of the separate contributions of the nest environment and earlier influences to development, and especially to reproductive performance.

METHODS

Animals

Mice were kept in two rooms, one at about 21° C and one at −3° C; both rooms were on a 12 hr light/dark cycle. There was no control of humidity. The cages were standard metal boxes with wire lids. The floors were covered with sawdust, and cotton wool was also supplied for nesting. Cages were cleaned, and bedding replaced, once a week, except that litters aged less than 10 days were left undisturbed. There was always an excess of a complete food (diet 41, Bruce & Parkes, 1949) and of water; at −3° C water was supplied as ice blocks, which the mice licked (Stoddart, 1966). Information on nest temperatures is given by Barnett (1956).

The mice were of the highly inbred strain A2G/Tb. The parents whose young were fostered, and which were used as foster-parents, were of three classes: (i) controls, at 21° C; (ii) 'new stock', of the first generation reared at −3° C; that is, their parents had been transferred to −3° C before breeding; (iii) 'old stock', of the seventeenth to twenty-third generations reared at −3° C. The old stock, though of the same inbred strain, were better adapted to the cold than the new stock (Barnett, 1965; Barnett & Little, 1965; Barnett & Widdowson, 1965).

Young of each class were fostered on to parents of each class; there were therefore

nine classes of fostered mice. Only second to fourth litters were used. Young were fostered within 24 hr of birth. The true young of the foster-parents were removed, and four young from the litter to be fostered were weighed as a group and immediately transferred to the foster-parents; the latter had always had their own litter within the previous 3 days. The fostered litters were also weighed as a whole at 10 days; and individual weights were recorded at 3 weeks. All weighing was to the nearest 0·1 g, on a Mettler direct reading balance. To discover whether there were any immediate effects of fostering, some litters of control and old-stock parents were reduced to four at birth, weighed, returned to their own parents, and weighed again at 10 and 21 days, after which they were not observed.

The fostered litters of each class of fostered mice were taken from their parents at the age of 3 weeks, and kept together to 5 weeks. Some males were thereafter kept in groups of three to five, and weighed at 5, 7, 10 and 16 weeks.

Whenever possible, one pair from each fostered litter was mated at 5 weeks. These pairs were kept together to the age of 28 weeks; if they then had an unweaned litter, they were kept until that litter was weaned. The births and sizes of their litters were recorded within 24 hr of birth; the young were weaned at 3 weeks, sexed and individually weighed. Some of these young were also mated, and their reproductive performance recorded in the same way. There were three of these second-generation classes: the young of old stock fostered on to controls, of controls fostered on to old stock and of controls fostered on to new stock. All were kept in the environment in which their parents had been reared.

The breeding performance of some non-fostered mice was also recorded at both temperatures. The conditions and recording procedures were identical with those of the fostered mice and their young.

Mathematical

Most of the statistical analysis was by computer. Comparison of means was by Student's t (two-tailed). On the main findings, on the reproductive performance of the nine classes of fostered mice, a preliminary two-way analysis of variance was carried out with equal cell sizes of nine each. When the results of these analyses of variance are referred to in the text, the degrees of freedom are in each case:

foster-parents	2
true parents	2
interaction	4
residual	72

In no case was there evidence of interaction.

RESULTS

The fostered mice up to weaning

Table 1 summarizes the observations on the fostered young to the age of 3 weeks. Reading down gives the influence of parentage, across shows the influence of foster-parents and their environment. 21° C → old and old → old gave the lowest mortality, but new → old the highest. The low mortality among two classes of mice fostered on to old stock conforms with the generally high performance of this stock (Barnett, 1961), but analysis of variance indicated no significant effect, either of true parentage or of foster-parentage, on mortality.

Much of the mortality was due to losses of whole litters. Accordingly, the percentage of litters lost was calculated for each class. This showed a marked influence of true parentage, resulting from high rates of loss among the litters born to the new stock. The mean for these three classes was $17{\cdot}0 \pm 2{\cdot}8$, that for the other six classes $6{\cdot}6 \pm 2{\cdot}1$ ($P < 0{\cdot}02$).

TABLE 1. Mortality (%) in nest of fostered mice, and weights (g) of fostered litters, at birth and 21 days. Means with their S.E.

True parents		Foster-parents: 21° C	−3° C (new)	−3° C (old)
21° C	No. of litters*	24 (22)	30 (29)	64 (61)
	Mortality	19·1	17·5	11·7
	Wt. at birth †	$6{\cdot}7 \pm 0{\cdot}16$	$6{\cdot}4 \pm 0{\cdot}08$	$6{\cdot}7 \pm 0{\cdot}16$
	Wt. at 21 days	$35{\cdot}1 \pm 2{\cdot}28$	$27{\cdot}8 \pm 1{\cdot}60$	$32{\cdot}0 \pm 1{\cdot}17$
−3° C new	No. of litters*	24 (19)	46 (39)	29 (24)
	Mortality	17·7	21·6	26·7
	Wt. at birth†	$6{\cdot}0 \pm 0{\cdot}15$	$6{\cdot}3 \pm 0{\cdot}10$	$5{\cdot}9 \pm 0{\cdot}09$
	Wt. at 21 days	$34{\cdot}4 \pm 3{\cdot}65$	$32{\cdot}9 \pm 3{\cdot}94$	$29{\cdot}3 \pm 1{\cdot}99$
−3° C old	No. of litters*	26 (22)	23 (22)	27 (26)
	Mortality	25·0	17·4	12·0
	Wt. at birth†	$6{\cdot}6 \pm 0{\cdot}31$	$6{\cdot}4 \pm 0{\cdot}16$	$6{\cdot}3 \pm 0{\cdot}16$
	Wt. 21 at days	$40{\cdot}5 \pm 0{\cdot}65$	$32{\cdot}7 \pm 1{\cdot}97$	$31{\cdot}3 \pm 1{\cdot}52$

* In parentheses, numbers surviving to 3 weeks.
† Each litter was reduced to four before weighing.

The mean weight of all new-stock litters at birth was lower than that of the controls ($P < 0{\cdot}001$) and of all old-stock ($P < 0{\cdot}01$). Hence the different growth characteristics of old stock and new stock (Barnett & Widdowson, 1965) influenced even prenatal development. Differences in litter weights at 3 weeks reflected principally the effect of environmental temperature during the nestling period: litters reared at 21° C were heavier than those reared at −3° C, regardless of parentage: the group mean for the three classes in the warm was 36·7 g, that for the six in the cold, 31·0 g ($P < 0{\cdot}02$).

Table 2 gives corresponding figures for control and old-stock mice of litters reduced to four at birth, but then left with their own parents. These may be compared with the figures for 21° C → 21° C and old → old in Table 1. There was evidence that fostering at −3° C had a slightly unfavourable effect on growth in the nest, as also reported by Butler & Metrakos (1950): old-stock litters fostered on to old-stock parents were lighter than old-stock litters that were merely reduced to four, weighed, and left with their own parents ($P < 0{\cdot}02$). The preceding statements apply to all litters, regardless of whether some members died before 3 weeks. A similar calculation was made for litters all of whose four members sur-

vived: the effect was then more decisive ($P < 0{\cdot}01$). There was, however, no evidence of severely adverse affects of fostering, such as those experienced by Albert, Wolf & Potter (1966) in strains C57BL and AKR.

TABLE 2. Mortality (%) in nest of mice not fostered, and weights (g) of non-fostered litters, at birth and 21 days. Means with their S.E.

	21° C	−3° C old
No. of litters*	22 (20)	21 (21)
Mortality	13·6	6·0
Wt. at birth	6·2 ± 0·13	6·3 ± 0·12
Wt. at 21 days	36·5 ± 2·14	37·3 ± 1·88

* In parentheses, numbers surviving to 3 weeks.

TABLE 3. Weights (g) of fostered male mice at 3 and 16 weeks, with mortality (%) between these ages. Means with their S.E.

True parents		Foster-parents: 21° C	−3° C new	−3° C old
21° C	Mortality	0·0	6·6	4·2
	No. of surviving mice	36	17	14
	Wt. at 3 weeks*	10·6 ± 0·22	8·6 ± 0·31	10·2 ± 0·54
	Wt. at 16 weeks	28·8 ± 1·26	27·3 ± 0·43	27·1 ± 0·74
−3° C new	Mortality	52·4	33·6	23·0
	No. of surviving mice	39	43	16
	Wt. at 3 weeks*	9·2 ± 0·28	9·3 ± 0·24	8·5 ± 0·26
	Wt. at 16 weeks	29·6 ± 0·43	27·0 ± 0·71	27·3 ± 0·26
−3° C old	Mortality	44·6	19·4	41·1
	No. of surviving mice	28	40	30
	Wt. at 3 weeks*	11·7 ± 0·34	9·8 ± 0·27	9·3 ± 0·24
	Wt. at 16 weeks	31·6 ± 0·51	29·1 ± 0·30	28·6 ± 0·39

* 3-week weights are only of the mice which survived to 16 weeks.

Mortality and growth, 3–16 weeks

Table 3 summarizes the findings for the male mice whose growth was recorded to 16 weeks. There was a marked effect of true parentage on survival: hardly any of the young of the controls (21° C) died between 3 and 16 weeks, while many of the others did. Nearly all deaths were before the age of 5 weeks; the death rates evidently reflected the ability of the young mice to survive weaning.

At 3 weeks the males that survived to 16 weeks weighed around 10 g, and at 5 weeks they had roughly doubled this weight. By 16 weeks they had added less than another 10 g, and most of this had accrued by 10 weeks. There were, however, differences between classes. Analysis of variance

reveals a contribution both of parents ($P < 0{\cdot}05$) and of foster-parents ($P < 0{\cdot}01$) at 3 weeks, and also at 16 weeks ($P < 0{\cdot}05$ and $P < 0{\cdot}01$, respectively).

As expected, weights tended to be higher at 21° C than at −3° C; this is shown by comparing 16 week weights in the left-hand column of Table 3 with the rest. The mean of the three classes at 21° C was $30{\cdot}0 \pm 1{\cdot}0$, and of the six classes in the cold $27{\cdot}7 \pm 0{\cdot}4$ ($P < 0{\cdot}03$). Growth was probably also favoured by having old-stock mice as true parents (bottom line of Table 3): the mean of these three classes was $29{\cdot}8 \pm 1{\cdot}1$, and of the other six, $27{\cdot}9 \pm 0{\cdot}5$ ($P < 0{\cdot}06$). Hence the heaviest mice were old → 21° C. Old-stock males had already been found to be heavier than new stock, and about the same weight as controls, at sixteen weeks (Barnett & Widdowson, 1965); evidently, there was still an effect of parentage even when mice were fostered at birth on to other strains.

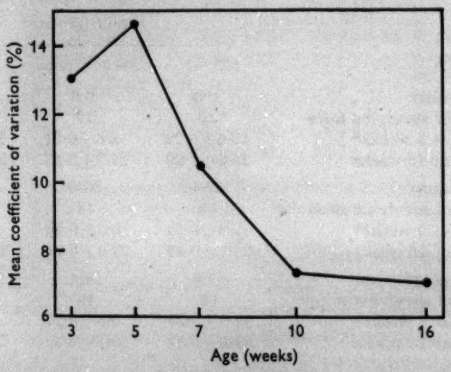

Fig. 1. Mean percentage coefficients of variation of the body weights of nine classes of male mice (Table 3) at five ages.

A notable difference between the weights at 3 weeks and those at 16 weeks was the lower variance at 16 weeks. The percentage coefficients of variation were therefore calculated for each age for which weights were recorded. The means of these for each age are shown in Fig. 1. There was a marked decline between 5 and 10 weeks. Evidently, the mice of each class tended towards a particular weight at the end of the period of major growth, and there was compensatory growth after weaning. The period during which growth was most likely to be thrown off course was the first 3 weeks after birth.

Reproductive performance of fostered mice

The main objective of these experiments was to assess the reproductive powers of the fostered mice. Table 4 summarizes the findings on the sizes of the litters born to them. These litters, unlike those that were fostered, were not culled at birth. Analysis of variance showed an effect of foster-parentage on litter size at birth ($P < 0{\cdot}01$) and at 21 days ($P < 0{\cdot}05$). There was no evidence of an effect of true parentage. The effect of foster-parentage was due to the higher litter sizes at 21° C than at −3° C: the figures in the first column of Table 4, which refer to the litters of mice reared at 21° C, are consistently higher than those in the other columns, which refer to the litters of mice reared at −3° C. The class mean for litter sizea t birth of the three classes at 21° C was $6{\cdot}3 \pm 0{\cdot}23$, and of the six classes at −3° C, $5{\cdot}3 \pm 0{\cdot}17$ ($P < 0{\cdot}01$). The corresponding figures for size of weaned litters are $5{\cdot}9 \pm 0{\cdot}19$ and $4{\cdot}9 \pm 0{\cdot}22$ ($P < 0{\cdot}02$). The total of young born per pair displays the same pattern (Table 5), and analysis of variance showed a similar effect of foster-parentage ($P < 0{\cdot}001$). There was no evidence of an effect of true parentage on number of young born.

TABLE 4. Young born per litter, and weaned per weaned litter, of fostered females. Means with their S.E.

True parents		Foster-parents: 21° C	−3° C new	−3° C old
21° C	No. of litters*	61 (55)	45 (34)	50 (39)
	Born/litter	$6{\cdot}1 \pm 0{\cdot}29$	$5{\cdot}2 \pm 0{\cdot}28$	$4{\cdot}6 \pm 0{\cdot}31$
	Weaned/litter	$5{\cdot}7 \pm 0{\cdot}25$	$5{\cdot}1 \pm 0{\cdot}30$	$4{\cdot}3 \pm 0{\cdot}29$
−3° C new	No. of litters*	102 (86)	69 (55)	50 (36)
	Born/litter	$6{\cdot}7 \pm 0{\cdot}23$	$5{\cdot}6 \pm 0{\cdot}20$	$5{\cdot}2 \pm 0{\cdot}28$
	Weaned/litter	$6{\cdot}2 \pm 0{\cdot}24$	$4{\cdot}2 \pm 0{\cdot}21$	$5{\cdot}2 \pm 0{\cdot}28$
−3° C old	No. of litters*	62 (58)	76 (70)	68 (63)
	Born/litter	$6{\cdot}2 \pm 0{\cdot}26$	$5{\cdot}5 \pm 0{\cdot}21$	$5{\cdot}6 \pm 0{\cdot}30$
	Weaned/litter	$5{\cdot}8 \pm 0{\cdot}28$	$5{\cdot}1 \pm 0{\cdot}25$	$5{\cdot}3 \pm 0{\cdot}28$

* Number weaned in parentheses.

The figures of losses of young between birth and weaning (Table 5) make a contrasting pattern. Analysis of variance shows an influence of true parentage ($P < 0{\cdot}01$), but no adequate evidence of an effect of foster-parentage ($P < 0{\cdot}2$). The effect of true parentage was due to the difference between the mice of the old stock and the rest: this is shown by comparing the figures in the bottom line of the Table, which refer to mice of old-stock parentage, with the percentage losses of the other six classes. The class mean for the former was $13{\cdot}0 \pm 1{\cdot}5$, and for the remaining classes $26{\cdot}0 \pm 3{\cdot}4$ ($P < 0{\cdot}05$). Of the six classes of which the two parents were not

old stock, only 21° C → 21° C had as low an infant mortality as those whose true parents were old stock. The percentage of whole litters lost corresponds: the class mean for mice of old-stock true parentage was $7{\cdot}3 \pm 0{\cdot}5$, and for the rest, $20{\cdot}0 \pm 2{\cdot}9$ ($P < 0{\cdot}02$).

If the three classes whose true parents were old stock are omitted, there is some indication also of an effect of temperature on infant mortality: the infant death rates of the remaining two classes at 21° C were much lower than those of the four classes at − 3° C; but these differences do not reach even the 10 % level of significance.

TABLE 5. Young born per pair of fostered mice, and losses between birth and weaning. Means with their S.E.

True parents		Foster-parents: 21° C	Foster-parents: − 3° C new	Foster-parents: − 3° C old
21° C	No. of pairs	14	13	15
	Young born	$27{\cdot}0 \pm 2{\cdot}58$	$18{\cdot}7 \pm 1{\cdot}40$	$15{\cdot}3 \pm 1{\cdot}08$
	% loss of litters	9·8	24·4	22·0
	% loss of young	$14{\cdot}5 \pm 4{\cdot}21$	$26{\cdot}3 \pm 4{\cdot}01$	$28{\cdot}8 \pm 4{\cdot}62$
− 3° C new	No. of pairs	20	21	15
	Young born	$34{\cdot}0 \pm 1{\cdot}40$	$18{\cdot}4 \pm 1{\cdot}37$	$17{\cdot}3 \pm 1{\cdot}38$
	% loss of litters	15·7	20·3	28·0
	% loss of young	$21{\cdot}2 \pm 2{\cdot}68$	$37{\cdot}2 \pm 4{\cdot}82$	$27{\cdot}7 \pm 5{\cdot}48$
− 3° C old	No. of pairs	12	20	19
	Young born	$32{\cdot}1 \pm 2{\cdot}28$	$21{\cdot}1 \pm 0{\cdot}98$	$19{\cdot}9 \pm 1{\cdot}50$
	% loss of litters	6·5	7·9	7·4
	% loss of young	$13{\cdot}4 \pm 3{\cdot}53$	$14{\cdot}9 \pm 2{\cdot}27$	$10{\cdot}8 \pm 2{\cdot}01$

The lack of correlation of nestling mortality with other measures of reproductive performance is also shown if reproductive performance is related to maternal body weight. Bluhm (1929) gives evidence of an effect of maternal weight on the birth weight of young mice; Bateman (1957) reports a positive correlation between maternal weight (even when recorded at 12 days) and milk secretion; McLaren (1960) states that maternal weight is positively correlated with litter size. Accordingly, we examined the relationship between the 3-week weight of fostered females and their reproductive performance. Within classes, there was no evidence of a correlation with number of young born, mean individual weights of young at three weeks or nestling mortality; but when correlations were calculated between class means there was evidence of a relationship. Mean 3-week weights of females of the nine fostered classes are given in Table 6. These means are positively correlated with the mean individual weights of the male young ($r = 0{\cdot}754$, $P < 0{\cdot}02$) and with those of the female young ($r = 0{\cdot}821$, $P < 0{\cdot}01$), also given in the Table. There is also a positive

correlation with mean number of young born (given in Table 5) ($r = 0{\cdot}697$, $P < 0{\cdot}05$). In contrast, there is no significant relationship of the mean weight of females with the mean mortality among their young ($r = 0{\cdot}257$).

TABLE 6. Three-week weights of fostered females and of the young of fostered females. Means with their S.E.

True parents		Foster-parents: 21° C	Foster-parents: −3° C new	Foster-parents: −3° C old
21° C	Number of ♀s	29	40	61
	3-wk wt. (g)	9·8 ± 0·27	8·1 ± 0·25	8·1 ± 0·20
	3-wk wts. of young (g) ♂	9·4 ± 0·13	7·7 ± 0·16	7·4 ± 0·13
	3-wk wts. of young (g) ♀	9·4 ± 0·14	7·3 ± 0·15	7·6 ± 0·14
−3° C new	Number of ♀s	36	77	43
	3-wk wt. (g)	8·8 ± 0·29	8·7 ± 0·15	8·3 ± 0·25
	3-wk wts. of young (g) ♂	9·1 ± 0·09	7·7 ± 0·12	7·7 ± 0·13
	3-wk wts. of young (g) ♀	8·7 ± 0·08	7·8 ± 0·06	7·9 ± 0·10
−3° C old	Number of ♀s	28	32	44
	3-wk wt. (g)	10·8 ± 0·27	8·6 ± 0·26	7·3 ± 0·67
	3-wk wts. of young (g) ♂	10·1 ± 0·10	8·4 ± 0·17	8·6 ± 0·11
	3-wk wts. of young (g) ♀	9·7 ± 0·10	7·9 ± 0·14	8·2 ± 0·12

TABLE 7. Breeding performance of offspring of fostered mice, compared with that of mice whose parents were not fostered. Means with their S.E.

	Young of old stock → 21° C: fostered	Young of old stock → 21° C: not fostered	Young of 21° C → new stock	First generation reared at −3° C	Young of 21° C → old stock
No. of pairs	12	11	10	10	10
No. of litters born (weaned)	62 (55)	52 (48)	31 (23)	26 (24)	30 (25)
Young born/litter	6·8 ± 0·31	6·1 ± 0·27	5·0 ± 0·39	5·5 ± 0·37	5·2 ± 0·38
Young weaned/weaned litter	5·7 ± 0·36	4·6 ± 0·25	4·9 ± 0·41	4·6 ± 0·35	4·6 ± 0·37
Young born/pair	35·3 ± 2·45	28·7 ± 2·05	15·6 ± 2·81	14·2 ± 2·80	15·6 ± 1·69
Nestling mortality (%)/pair	25·2 ± 4·24*	30·4 ± 3·76	26·7 ± 8·26	19·3 ± 6·25	27·3 ± 4·78

* Higher than *parents* ($P < 0{\cdot}05$).

Reproductive performance of young of fostered mice

Table 7 summarizes the breeding performance of the *offspring* of three classes of fostered mice. It also compares them with mice whose parents had not been fostered, but had otherwise undergone similar changes of environment. The three right-hand columns refer to mice bred at −3° C. Column 4 gives findings on mice whose *parents* had been transferred to −3° C on mating at the age of 5 weeks; columns 3 and 5 give figures on mice whose parents had been born to controls at 21° C and fostered on to

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females at −3° C. None of the differences between them is statistically significant. There is, accordingly, no evidence of an effect on this generation of the rearing of their parents by either of the types of foster-parents which had grown up in the cold environment.

The two left-hand columns of Table 7 compare two classes reared at 21° C. Here there is evidence of an effect of the fostering of the parents: litter size at birth of the mice with fostered parents was the higher of the two groups ($P < 0{\cdot}02$), and correspondingly the number of young born per pair was also higher ($P < 0{\cdot}05$). The mothers of the mice whose record is given in column 1 were reared from birth in the warm environment, and their foster-parents were members of the colony kept at 21° C; by contrast, the mothers of the mice whose record is given in column 2 were reared by their own parents and remained to the age of 5 weeks in the cold environment. The superior reproductive performance of the first group is therefore attributable to a favourable effect on their mothers of being reared at 21° C.

It might be expected that any such effect would also be reflected in the pre-weaning mortality of the young. Mortality did differ in the expected direction, but the difference was not statistically significant. The mortality of nestlings whose grandparents had been fostered reflected, in fact, the environment in which the nestlings were reared: it resembled that of the mice kept permanently at 21° C (Barnett, 1961), and was nearly twice that of the parental generation (recorded in Table 6). Hence the effects on nestling mortality of long ancestry in the cold, evident among the mice fostered on to parents at 21° C, did not persist in the second generation in the warm environment.

TABLE 8. Weights (g) at the age of 3 weeks of the young of three classes of mice at 21° C. Means with S.E. N = number of mice

	21 → 21° C (*a*)		Old stock → 21° C (*b*)		Offspring of (*b*) (*c*)		Difference of (*b*) from (*c*)
	N		N		N		$P <$
♂	161	9·4 ± 0·13	249	10·1 ± 0·10	155	9·0 ± 0·25	0·001
♀	147	9·4 ± 0·14	242	9·7 ± 0·10	169	8·7 ± 0·12	0·001

The absence, at 21° C, of any lasting influence of old-stock ancestry was also evident in the weights of the young aged 3 weeks. Table 8 gives the weights of the young of two classes of fostered mice at 21° C: the young of old-stock mice fostered on to controls (old → 21° C; column *b*) were heavier than those of controls fostered on to controls (21 → 21° C; column *a*); the difference was statistically significant only for the males ($P < 0{\cdot}001$). The offspring of the young of old → 21° C mice (column *c*) were, however, lighter, and resembled those of the 21 → 21° C mice.

A final question was whether the old stock, nevertheless, retained their special properties after transfer to 21° C, but in a latent form. Non-fostered old-stock mice were therefore transferred to 21° C at mating, and bred there. Some of their young were returned to − 3° C at mating. Table 9 compares the breeding performance of the young of these re-transferred mice with that of old-stock mice, breeding at − 3° C at the same time, with an unbroken ancestry of twenty generations in the cold. The re-transferred mice had a high nestling mortality and showed no evidence of a lasting effect of their ancestry.

TABLE 9. Breeding performance of old-stock mice (not fostered) at − 3° C. Means with their S.E.

	Permanently in cold*	After transfer†
No. of pairs	32	12
Young born/pair	21·7 ± 1·28	22·6 ± 2·17
Nestling mortality (%)/pair	13·9 ± 1·93	32·6 ± 3·48

* 20 generations of unbroken ancestry in cold.
† Young of old-stock mice whose parents had been bred at 21° C.

DISCUSSION

When these experiments were planned we expected a marked effect of foster-parentage on the fostered nestlings, and especially on their survival. In the outcome, survival was significantly influenced only by true parentage: between birth and weaning losses of whole litters born to new-stock mothers were much higher than among those born to mothers of the other two classes. The new-stock mice were also slightly lighter at birth than the rest. The only immediate effect of conditions in the nest was that of temperature on growth: at 3 weeks, litters in the warm were consistently heavier than those at − 3° C. The different treatments would have had greater effects had the fostered litters been larger: the differential influence on litter weight of environmental temperature is most evident among large litters (Barnett & Neil, 1971), that is, when there is competition for milk (Falconer, 1947; Bateman, 1957; Ashoub, Biggers, McLaren & Michie, 1958) or perhaps for some other aspect of parental attention (Barnett & Burn, 1967).

The observations on the fostered young between birth and 3 weeks show a lack of correlation between two important features of early development, namely, survival and growth: in our experiments, survival was especially influenced by true parentage, that is, presumably by conditions *in utero*, while growth reflected the immediate environment of the nest. The effect

of true parentage on survival was also evident after weaning, among the mice kept to sixteen weeks for study of body weight. Monteiro & Falconer (1966) analysed maternal effects, defined as all environmental influences shared by the members of a litter, on the growth of mice: in their experiments the variance due to this source was substantial up to the age of 4 weeks, but then declined sharply during the next 4 weeks. They also observed compensatory growth: increase in weight after 4 weeks was inversely related to weight at that age. Similarly, the weights of our mice up to 16 weeks displayed compensatory growth; at 16 weeks they also reflected, more clearly than at 3 weeks, the influence of true parentage, since those whose true parents were old stock were heavier than the rest in both temperatures.

All the mice of this study were of the same highly inbred strain. Accordingly, the simplest hypothesis to account for the influence of true parentage is to invoke a lasting effect of the uterine environment. Since this was reflected in adult growth, it was likely that it would also be evident in the reproductive performance of the fostered mice. The experimental design allows some differentiation of the effects of (i) temperature of rearing, (ii) the two kinds of foster-parentage at $-3°$ C, and (iii) true parentage.

(i) Litter size, especially at birth, and the closely correlated young born per pair, were influenced by the temperature in which the parents had been reared and were breeding: they were consistently higher at 21° C than at $-3°$ C. The same applied to the weight of the young at 3 weeks, in spite of the slightly larger litters, and consequently greater competition among litter mates, in the warm.

(ii) At $-3°$ C there was no evidence of differences in reproductive performance due to foster-parentage: hence, whatever differences there were in the maternal environments provided by the two classes in the cold, those that acted post-natally evidently did not influence reproduction.

(iii) The most notable differences between classes were in nestling mortality. It might be expected that, of all the features recorded, this would be most affected by temperature; but the principal separation between classes was between those of old-stock true parentage and the rest (Table 5). The number of young born, per litter or per pair, did not correspond, and so there was probably no effect of old-stock parentage on the survival of young *in utero*. Hence the hypothesized lasting effect of the uterine environment on the reproductive performance of females manifested itself only in the rate of survival of their young in the nest.

A gradual decline in nestling mortality during several generations in the cold first drew attention to the peculiarities of our old-stock mice; in accord with our present findings, this decline was not accompanied by any change in number of young born (Barnett, 1961). There is no plausible

genetical mechanism for a change of this kind in a highly inbred strain, and so a cumulative maternal effect has been proposed. Among possible maternal influences is a secular change in the relationship of a population with an infective agent (Barnett, 1961, 1965). Grüneberg (1970) has proposed a similar hypothesis to account for certain differences between substrains of inbred mice, especially those of the apparently rather unstable C57BL group. He suggests extra-nuclear inheritance, due to virus-like particles, which could simulate the effects of mutation.

A change from the latter cause would persist in subsequent generations, but the change in our old stock mice was not stable in the warm environment. Evidently, the special characteristics of these mice depended on development in a cold environment, at least until just after birth (Barnett & Borland, 1967): on transfer to 21° C they reverted to the control pattern in the second generation; and return to −3° C after one generation at 21° C did not restore their peculiar features. It is therefore still not possible to propose a simple hypothesis to account for the effects of continued breeding at −3° C. The problem may be at present insoluble. Our present findings do, however, suggest the need for more attention to two phenomena. One is the causes of mortality, especially the loss of whole litters, in the nest. The other, more important, is that of maternal effects in which only three generations are involved.

This work was supported by grants from the Medical Research Council and the Royal Society. We are also greatly indebted to R. A. Elton for statistical advice, and to Margaret J. Little for help with the computations.

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J. Physiol. (1971), **215**, *pp.* 679–692
With 1 *plate and* 6 *text-figures*
Printed in Great Britain

INTERACTION BETWEEN COLOUR AND SPATIAL CODED PROCESSES CONVERGING TO RETINAL GANGLION CELLS IN GOLDFISH

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(*Received 15 October 1970*)

SUMMARY

1. Extracellular recordings were made from ganglion cell units in the isolated goldfish retina. The discharge patterns of these units are spatial *and* colour coded.

2. Even for weak stimuli these ganglion cell responses are highly distorted. To a first approximation a rectifying element can account for the observed distortion.

3. A method is introduced to determine whether the interaction between the spatial and colour-coded processes occurs preceding or after the rectifying stage.

4. With various stimulus patterns such as checkerboards, bars, annuli, etc., not only the location of the interaction point but also the mode of operation underlying the colour and spatial interactions has been studied.

5. The presented data indicate that for the common phasic ganglion cells in the goldfish retina spatial and colour interaction occurs preceding the rectifying stage. Moreover an algebraic mode of operation governs this interaction.

6. The charm of the presented method lies in the fact that no assumptions need to be made about the dynamics of the various retinal transformations converging to the ganglion cells. Neither is a description required for the non-linear (rectifying) element in order to answer the question of the location and the mode of operation of the colour and spatial interaction mechanism.

INTRODUCTION

The discharge pattern of the ganglion cell responses to illumination of the goldfish retina is a function both of the colour and of the spatial configuration of the stimulus. The ganglion cell response which is most frequently found in this retina consists of a short burst of spikes to the onset

or the cessation of the stimulus light. The most common type of these so-called 'phasic' units gives an 'off' response to stimuli whose wave-lengths are at one end of the spectrum and an 'on' response to stimuli in the other end of the spectrum (Wagner, MacNichol & Wolbarsht, 1960). Red 'on'–green 'off' and red 'off'–green 'on' processes can be distinguished in the centre of the receptive field. Surrounding processes can be revealed by stimulating such a ganglion cell with an annulus concentric to the electrode (Daw, 1967). The colour codings of centre and surrounding processes have been reported to be identical but the 'signs' of the surrounding processes are opposite to the 'signs' of the centre processes. If, for example, an 'on' response is obtained by stimulation with a red spot, an 'off' response will be found when a red annulus is used.

Many articles have been published in recent years about the interaction between the two mutually antagonistic, concentrically organized centre and surround regions. Most of these experiments have been performed on the retinal ganglion cells in the cat. It has been shown that the ganglion cell responses in this retina reflect a linear operation in the integration of the signals coming from antagonistic regions (Enroth-Cugell & Pinto, 1970). However, to a first approximation, the ganglion cells in the cat retina behave linearly when centre and surround processes are either simultaneously or separately stimulated (Maffei & Cervetto, 1968). They produce sinusoidal outputs when sinusoidally driven (Hughes & Maffei, 1966).

This is not the case for phasic retinal ganglion cells in the goldfish. Here, a strongly distorted response is evoked by stimulation with sinusoidally modulated light. The non-linear behaviour of these ganglion cells, even when weak stimuli are used, furnishes direct evidence for the existence of an essential non-linear element in the chain of retinal transformations in goldfish. Therefore the spike responses of the goldfish retina cannot be usefully described by the methods of linear system analysis.

In this paper a method will be presented to answer the question whether the spatial integration in the goldfish retina occurs before or after the essential non-linearity. In addition an account is presented of an investigation of the operation of the mechanisms that underlie both the spatial *and* colour interaction of the retinal processes converging to the goldfish ganglion cells.

METHODS

Common goldfish (*Carassius auratus*) measuring between 15 to 20 cm long were used in all experiments. The animals were kept in a dark room for at least several hours before the experiment. The eye was quickly removed from the head, opened, and the dark-adapted retina was lifted out after separation from the optic disk. Almost certainly some loss of rod outer segments occurred. The receptor side was usually fairly free of melanin pigment.

The retina was spread, receptor side up, upon a piece of glass in a moist chamber. The chamber was kept at 15° C and moist gas (95 % oxygen, 5 % carbon dioxide) passed over the preparation. Light for stimulation came from below, passing through the layers of the retina in normal direction. The micro-electrode entered from above.

The retina was stimulated by patterns of illumination generated by an optical stimulator which provides control of spatial configuration, wave-length and intensity. The light sources were two electronically controlled television projection tubes; Philips, MW 6/2 (Denier van der Gon, Strackee & van der Tweel, 1958). The phosphors had broad emission spectra and gave a colour temperature of approximately 6500° K. The intensities of the two light sources could be modulated; photocells monitored and controlled the outputs of the projection tubes. The peak-to-peak value of the spontaneous intensity fluctuations was smaller than 1 ‰ of the mean intensity level. The stimulus intensity was limited to a maximum of about 2·5 log units above threshold for units in the goldfish retina.

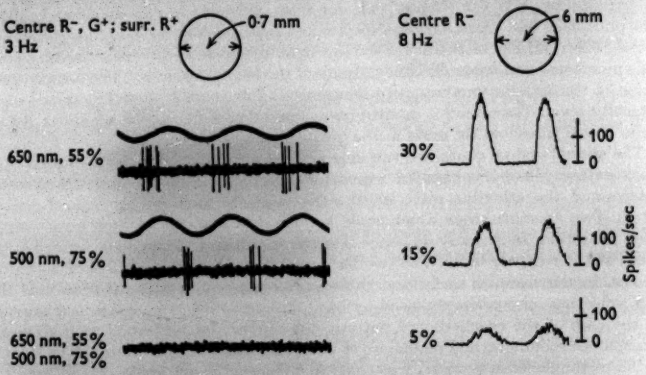
The optical system contained two independent but essentially identical pathways. Each pathway had a collimated region for placement of interference filters which determined the stimulus wave-length; the intensity was set by neutral-density filters. Two aperture stops which could be varied in size and position were focused upon the retina. In this way an image of spots, annuli and slits could be formed at the plane of the receptor layer. The two pathways were combined by a 50 % reflecting mirror. In the stimulus situations where more complicated patterns were used this half reflecting mirror was replaced by bar-, checkerboard-, etc., patterned mirrors. In the case of the checkerboard pattern, the mirror was patterned with alternate transparent rectangles and rectangles of reflecting material. The ratio of the lengths of the rectangle sides were $1:\sqrt{2}$ so that when focused at an angle of 45° upon the retina, the mirror appeared to be patterned with squares. With the two light sources modulated in counterphase at equal intensities an alternating checkerboard was generated. In all situations the patterns were focused on the retina by viewing through a dissecting microscope; they were not noticeably distorted by passing through the retinal tissue, as can be seen in Pl. 1.

Recordings of the ganglion cell spike potentials were made with platinum–iridium micro-electrodes (Wolbarsht & Wagner, 1963). The indifferent electrode was a platinum wire placed in contact with the retina at some convenient point. The signals from the micro-electrode, which was centred under direct microscopic observation with respect to the stimulus field, were detected through conventional electronic amplification. The output of the amplifier was displayed, together with the signals of the photocells, on an oscilloscope. In order to improve the signal-to-noise ratio the responses, fed through a pulse shaper, were analysed by a computer of average transients (CAT). The number of summations was generally 50–600, depending upon the ganglion cell discharge rate. A final record was obtained by plotting the analogue output of the computer with an x–y recorder.

RESULTS

Initially, square-wave stimulation with a frequency of 0·5 Hz and 100 % modulation was used in order to isolate and classify a unit according to its colour and spatial coding. Next, other wave forms and stimulus configurations were applied in order to investigate the mechanism underlying the colour and spatial interaction. Sinusoidally modulated light was employed as a tool to detect the non-linear behaviour of the phasic ganglion cells in the goldfish retina.

The left column of Text-fig. 1 gives an example of such a response. This Figure shows that sine-wave stimulation produces a burst of action potentials only once during each stimulus cycle. For the red–off, green–on unit shown, stimulation with a sinusoid of 3 Hz resulted in discharges that were roughly in counterphase for the two colour-coded responses. The right



Text-fig. 1. Ganglion cell responses to sine-wave-modulated light. The degree of modulation is expressed as a dimensionless parameter, $m = A/L_o$, in which A is the amplitude of the sine wave and L_o is the mean intensity of the stimulus signal. The parameter m varies between 0 (0 %) for unmodulated and 1 (100 %) for fully modulated light.

Left column gives an example of the spike responses to sinusoidally modulated light of 3 Hz. The centre response of this particular cell (red–off, green–on centre) to a red sine-wave stimulus is roughly in counterphase with the response to an identical green coloured light. The bottom row shows that simultaneous stimulation with both coloured light does not result in any spike discharge at all. The mean intensity of the stimulus passed through Ealing-TFP interference filters is approximately 6 μW/cm^2 for the red and 20 μW/cm^2 for the green stimulus.

Right column gives the average spike response of a red–off centre process as a function of the modulation depth. It should be noted that even at 5 % modulation the response is still highly distorted (half-wave rectified). The mean intensity of the stimulus passed through a Wratten 29 filter is approximately 1 μW/cm^2. The number of summations with the CAT computer amounts to 300.

column of Text-fig. 1 gives the average response of a red–off unit. For half the stimulus period the density distribution of the nerve impulses had roughly the same shape as the sinusoidal stimulus, but during the other half of the stimulus cycle no spike discharge could be observed at all. The amplitude of the average response is approximately proportional to the strength (modulation depth) of the sine-wave stimulus. These data suggest that a

half-wave linear rectifier can model the distortions observed in the discharge pattern of the phasic ganglion cells to 'small' signal stimulation (see also Spekreijse, 1969).

Spatial interaction for a centre colour-coded process

In order to study the spatial interaction within the centre of the receptive field the retina was stimulated with a checkerboard pattern. Sine-wave modulation of exclusively one set of squares gives a response that, according to the data in Text-fig. 1, may be compared to a first approximation with a half-wave rectified sinusoid. Counterphase modulation of the other set of stimulus squares with the same mean intensity and modulation depth as the first set results, of course, in the same half-wave rectified sinusoid response shifted 180° in phase. Simultaneous counterphase stimulation of both sets of checkerboard squares could give one of the following two responses (Text-fig. 2): either a full-wave rectified sinusoid or no spike discharge at all. This can be understood as follows: if the spatial summation for a centre process takes place *after* the essential non-linear element, such as a half-wave rectifier, then the response would consist of a full-wave rectified sinusoid with the same amplitude as the original half-wave rectified response (Text-fig. 2*a*). On the other hand, if spatial summation occurs at a site *preceding* the non-linear element, then the two sets of input sinusoids counterbalance each other completely; and no response results (Text-fig. 2*b*). Text-fig. 2(*c*) gives the actual data for a green 'off' centre process. As is evident from this Figure, no spike discharge is observed when the two sets of squares are modulated simultaneously in counterphase. This holds, of course, only if the strengths of the responses to each set of squares are identical.

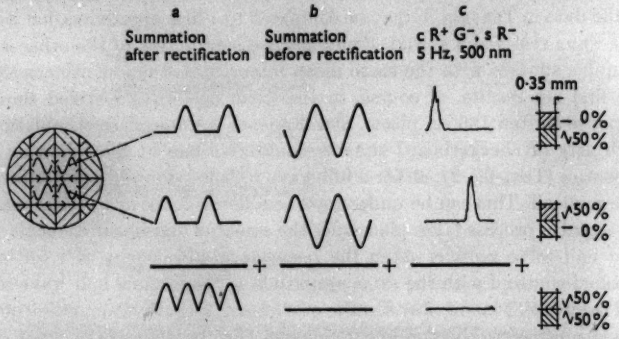
Neither the type of pattern nor the sizes of the pattern elements proved to be of importance in these experiments. Repeating the above experiments with bar as well as checkerboard patterns with various widths and square sizes, gave always similar results, as the ones depicted in Text-fig. 2*c*. Therefore the data demonstrate directly that in the goldfish retina the spatial interaction for a colour-coded centre process takes place preceding the essential non-linear element.

Interaction between colour-coded centre processes

As pointed out in the introduction, the type of ganglion cells most frequently found in the goldfish retina exhibits two antagonistic colour-coded centre processes: red and green. The spectral characteristics of each of these processes closely resemble the absorption spectra of the cones (Witkovsky, 1965). This indicates that cone outputs are grouped according to their

colour coding, and that the output signals of the two sets of cones travel separately through the retinal layers, or at least are only partly fused.

In order to investigate the functional location of the summing point between the red and green centre processes, we stimulated the retina with a red modulated light superimposed on a constant green background or vice versa. For each modulating signal, red respectively green, a modulation depth was chosen so that the responses were identical in size independently of the stimulus colour. An additional phase shift was necessary to have the



Text-fig. 2. Schematic representation (columns *a* and *b*) of retinal modes of summation between counterphase signals generated by the two sets of counterphase modulated squares of a checkerboard pattern.

Column (*a*) demonstrates that the summed response consists of a full-wave rectified sinusoid in the case that the retinal interaction takes place after the rectifying element.

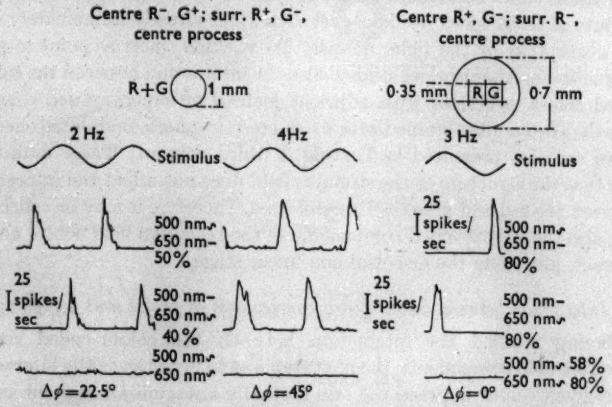
Column (*b*) shows that no response will be obtained in the case that the spatial summing of the two counterphase signals occurs preceding the rectifying element. This holds of course only if the strengths of the responses to each set of squares are identical.

Column (*c*) gives the actual data for a green–off centre process. The top figure is the average spike response to modulation (50 %) of one set of squares (0·35 mm width). The other set of squares is not modulated at all but has a steady intensity identical to the mean intensity of the modulated set. Reversing the condition and modulating the other set of squares with the same depth, but in counterphase, results, of course, in the same rectified sinusoid but shifted 180° in-phase (mid figure). Simultaneous counterphase modulation of both sets of squares does not result in a spike discharge at all (bottom figure); an observation which suggests a location of the summing point at a stage preceding the rectifier.

The mean intensity of the stimulus light passing through an Ealing FTP interference filter is approximately 4 μW/cm^2. The number of summations is 100; the modulation frequency is 5 Hz. The patterned spot focused on the retina has a diameter of 2 mm. For this particular unit a spot of even larger diameter could have been used, since no green colour-coded surrounding process could be detected.

individual responses to each stimulus colour exactly in counterphase. As can be seen in Text-fig. 3 (first column) under such condition simultaneous modulation of both coloured stimulus lights did not give a spike discharge (see also Text-fig. 3, left column).

Since twice this phase shift was required to cancel the two colour-coded responses when the stimulus frequency is doubled (Text-fig. 3, second column), this could suggest a latency difference between the red and green



Text-fig. 3. The Figure demonstrates the interaction between two different colour-coded centre processes. The left half Figure gives the average responses to two superimposed coloured spots. The first column gives the responses for a modulation frequency of 2 Hz and the second column for a frequency of 4 Hz. The right-hand column gives the responses to adjacent instead of superimposed coloured fields. In this case a modulation frequency of 3 Hz has been chosen. In all three columns the top row gives the stimulus. The second row gives the average responses to the modulated green fields (red fields steady) and the third row presents the average responses to the modulated red fields (green fields steady). In each column, except the third, the modulation depths of the red respectively green stimuli are chosen so that the average responses are identical in size, irrespective of the colour used. Under such a condition simultaneous in-phase modulation of both coloured stimulus fields does not result in a spike response (bottom row). To obtain this result, generally an additional phaseshift ($\Delta\phi$) is required so as to have the responses to each of the coloured lights exactly in counterphase. For the superimposed condition the mean intensity of the 500 nm stimulus is approximately 2 μW/cm^2 and the intensity of the 650 nm stimulus is 0·6 μW/cm^2. For the adjacent field condition these values are respectively 9 and 1·2 μW/cm^2. The number of summations is 50 for the 2 Hz stimulus and 100 for the 4 Hz and 3 Hz stimuli. Calibration bars give the number of spikes/sec; the zero spike level is given by the lowest address points.

colour-coded centre process.* However, for high stimulus frequencies (> 8 c/s) the required phase shift difference between the red and green colour-coded processes was no longer proportional to the stimulus frequency. The origin of the phase difference remains therefore a point of further investigation.

It is well known that a large percentage of the cones in the goldfish retina are so-called double cones (Marks, 1965). It could be possible that only these cones contribute to the red–green centre processes. If so, the outputs of these cones would cancel each other already immediately after the absorption of the light quanta. To exclude such a point-to-point interaction mechanism, we studied also the interaction between the colour-coded centre processes with adjacent instead of superimposed coloured stimuli. The results obtained with a red–green in-phase modulated checkerboard are also presented in Text-fig. 3 (third column). These data indicate that the structure of the stimulus field does not affect the interaction between the red and green centre processes. Therefore it may be concluded that also the colour-coded components of the receptive field centre always interact, preceding the essential non-linear stage.

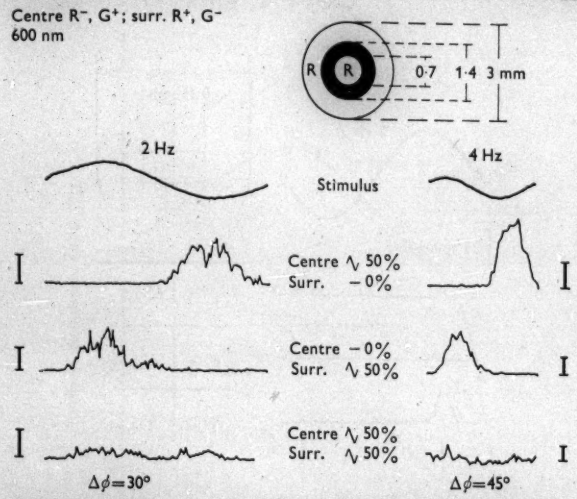
Interaction between colour-coded components of centre and periphery

Having studied the interaction between the colour-coded central receptive field components, the next step was to investigate the interaction for a given colour between the two mutually antagonistic regions: centre and periphery. With *one* colour e.g., red, we stimulated centre and periphery simultaneously. The stimulus configuration consists of a central disk surrounded by an annulus. The two stimulus fields are not adjacent to reduce possible effects of scattered light. With steady illumination of one of the two stimulus fields we determined for centre and periphery separately the modulation depth required to obtain equally sized responses. Next we chose a phase shift between the central and peripheral stimulating signals such that the two responses themselves were in counterphase. Simultaneous stimulation under such a condition of the central and peripheral processes gives very weak spike discharges. This is illustrated in Text-fig. 4 for a red 'off' centre, red 'on' surrounding type unit. It follows that for central and peripheral processes also summation occurs, preceding the essential non-linear stage. Our experimental finding is that this holds not only for identical colour-coded components, but also for interactions between, for example, green-centre, red-surrounding processes.

* Phase shift and latency are related to each other with the frequency as the proportionally factor ($\phi = -\omega\tau$).

Dynamics of the interactions mechanism

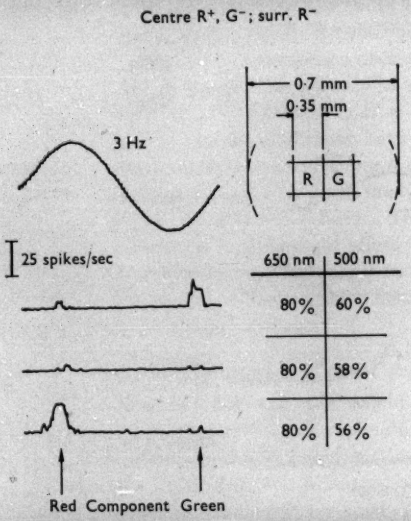
Up till now we have paid attention mostly to the location of the spatial interaction point in the chain of retinal transformations. However, the data presented here indicate not only that the spatial *and* colour interaction occurs preceding the essential non-linear stage, but also suggest an



Text-fig. 4. Interaction between the two mutually antagonistic regions: centre and periphery. The responses are from a red–off centre, red–on surrounding unit. Modulation of either the centre spot (annulus steady) or of the concentric annulus (spot steady) results in a highly distorted (rectified) response. Simultaneous in-phase modulation of both the centre spot as well as the annulus gives a very weak spike discharge (bottom row). An additional phase-shift ($\Delta\phi$) is needed to have the individual responses exactly in counterphase. Generally it is more difficult to obtain a zero spike response for this condition than for any of the previous situations. The smaller strength of the 2 Hz response in comparison to the size of the response to the 4 Hz stimulus suggests a low-frequency attenuating process. Such a differentiating process is characteristic for phasic units. The mean intensity of the centre spot amounts to 15 μW/cm^2; the intensity of the annulus is 30 μW/cm^2. The number of summations is 25; the calibration bars are 100 spikes/sec. The zero spike level is given by the lowest address points.

algebraic mode of interaction. This follows directly from the observation (Text-fig. 5) that only subtractive interaction of *equally sized* responses results in a zero response. The slightest difference in the sizes of the subtracting signals results in a spike discharge. The data of Text-fig. 5

illustrate the sensitivity of this zero-setting for the red–green interaction mechanism. A modulation depth increase or decrease of no more than 2% for the green stimulus resulted in a clear dominance of the green or the red component in the spike response. Such a sharp minimum means that the positive and negative halves of the stimulating sinusoid are treated



Text-fig. 5. The slightest difference in the strengths of the responses to the green, respectively red modulated set of checkerboard squares results in a disturbance of the zero-spike-response setting. The Figure shows that, keeping the modulation depth of the red stimulus constant, a 2% increase of the modulation depth of the green stimulus light results in a dominance of the green component in the final response. Similarly, a 2% decrease of the modulation depth of the green stimulus light results in the dominance of the red component of the final response. The unit from which these data are taken is the same as the one depicted in the right-hand column of Text-fig. 3. For the stimulus settings see the legend of that Figure.

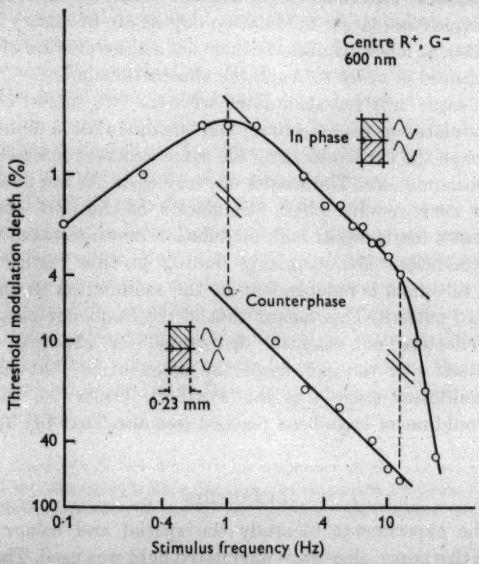
identically by the retinal processes preceding the interaction point. Therefore for weak stimuli an approximately linear input–output relationship holds for the primary processes; suggesting an algebraic mode of operation for the spatial and colour interaction.

Saturation

In applying the checkerboard method we assumed that only *one* non-linearity accounts for the distortion in the ganglion cell responses. As the

observations in, for example, Text-fig. 2(*c*) indicate, this is practically true; but only to a first approximation! In reality none of the elements in the chain of retinal transformations behaves linearly. Any element has a restricted dynamic range and, when driven sufficiently strongly, each of them will exhibit non-linear behaviour such as saturation.

In terms of Fourier analysis this means that early elements may generate



Text-fig. 6. The so-called de Lange curves for a red–on centre process. The modulation depth required to reach a constant threshold criterion is plotted as a function of the frequency of the sine-wave modulated checker-squares. The upper curve gives the threshold modulations for in-phase modulation of the two sets of squares. The bottom curve represents the data points for counterphase modulation of these two sets. The mean intensity of the 600 nm stimulus is approximately 0·4 μW/cm^2. The diameter of the circular patterned spot focused on the retina is 1·5 mm; the width of the checker squares amounts to 0·23 mm.

harmonics. If such harmonics (in particular even harmonics) are formed at a stage *preceding* the summing point of the receptive field centre, then they can *never* be cancelled by counterphase stimulation of the two sets of checkerboard squares. This offers the possibility of determining at which modulation depth distortion occurs early in the system, i.e. before the 'rectifying' stage. The results of this experiment are presented in Text-fig. 6.

The upper curve in this Figure is the so-called de Lange curve. This curve is determined for a red 'on' centre ganglion cell by in-phase stimulation with identical settings of the two sets of checkerboard squares; thus no structure is present in the stimulus plane. For a constant mean intensity the de Lange curve represents the modulation depths requested as a function of the sine-wave frequency to reach an arbitrarily chosen, constant-response criterion. As is evident from the curve, for higher stimulus frequencies, larger modulation depths are necessary to reach the criterion. Also, at lower stimulus frequencies some elevation of modulation depth is required in order to reach the chosen threshold.

Next the same unit was stimulated with the two sets of checkerboard squares modulated in counterphase, and the modulation depth was determined to reach the threshold using the same constant intensity as for the in-phase measurements. The results are very clear. As can be seen in Text-fig. 6, lower curve, counterphase stimulation of the two sets of squares needs at least a ten times as high modulation depth to reach the in-phase threshold condition. The surprising finding in this Figure is that the increase in threshold is independent of the modulation frequency of the checkerboard pattern. This means that in the frequency range measured the first effective non-essential distortion (for example, saturation) manifests itself *after* the high-frequency attenuation, but *preceding* the essential non-linear stage (i.e. the rectifier). Otherwise the threshold criterion would never have been reached (see also Text-fig. 3).

DISCUSSION

In all the experiments to study the spatial and colour interaction reported in this paper, sine-wave modulated light was used. The advantage of this stimulation above any other type of stimulating signal lies in the fact that application of this stimulus does not require any knowledge about the dynamic characteristics of the interacting processes. For example, if these characteristics differ, the responses of the interaction processes can never be equalized for any other type of stimulus without changing the modulating wave forms. In contrast, equal responses can always be obtained with sinusoidal stimulation merely by choosing the correct amplitudes and phases of the input sinusoids.

In addition, the zero-response method described in this paper has an advantage over the commonly used phasor representation to study the interaction mechanism (Maffei & Cervetto, 1968). As discussed already by Enroth-Cugell & Pinto (1970), the phasor technique can be successfully applied only if precise assumptions are made about the characteristics of the non-linear process involved. This is not so for the zero response method.

As is illustrated in this paper, this method can answer the question of the mechanism underlying the spatial *and* colour interaction without the necessity of a description of the characteristics of the (essential) non-linearities involved.

Not all ganglion cells in the goldfish retina are silent when no stimulus is applied. When such units are sinusoidally modulated, their dynamic behaviour is similar to that of the ganglion cells in the cat retina. The observations presented here hold also for these ganglion cells, but for moderate modulation depths the essential non-linearity is masked by the spontaneous activity (Spekreijse, 1969). Therefore for these units the location of the interacting point cannot be determined by the methods described in this paper; only the mode of operation can be investigated. Our observations indicate that for the spontaneous units also an algebraic operation underlies the colour *and* spatial interaction.

The results described here do not give any specific answers to how the coding of the sensory information is at the stage where the colour and spatial interaction occurs. However, taking into account the present concepts about spike-generating mechanisms, it seems unrealistic to suppose that at the interaction point the nerve impulses are already generated. Only direct investigation from the nuclear layer between photoreceptors and ganglion cells can give an answer to this question. Such a further investigation is also needed to determine at which pre-ganglion level the interaction occurs.

We wish to thank Mr N. Schellart for his assistance in the experiments and the preparation of the manuscript. This research was supported by a grant (90-6) from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.)

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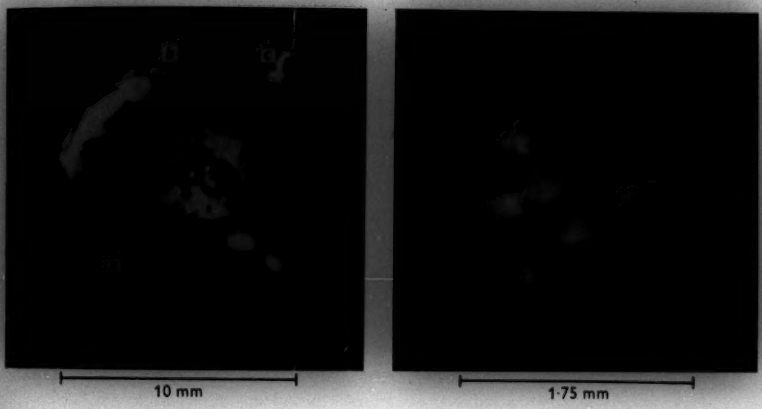
EXPLANATION OF PLATE

Fig. 1. The left-hand photograph shows an isolated retina in the moist chamber. A circular light spot is projected from below on to the retina (*a*); the micro-electrode (*b*) enters from above; the retina is placed in contact with the indifferent electrode (*c*). The circular spot has a diameter of 5 mm. The right-hand photograph illustrates that a checkerboard pattern focused on the retina is not much distorted by passing through the retinal tissue. The circular patterned spot has a diameter of 2 mm; the width of the checker-squares amounts to 0·35 mm.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J. Physiol. (1971), **215**, *pp.* 693–707
With 9 text-figures
Printed in Great Britain

THE INFLUENCE OF DEEP BODY TEMPERATURES AND SKIN TEMPERATURES ON PERIPHERAL BLOOD FLOW IN THE PIG

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(*Received 23 October 1970*)

SUMMARY

1. The rate of blood flow through the tail of the pig has been measured by means of venous occlusion plethysmography using a mercury in rubber strain gauge. Conscious animals were used in all experiments because previous work had demonstrated that anaesthetics interfere with the animal's ability to vasoconstrict.

2. Graded changes in the temperature of the hypothalamus were imposed by means of an implanted thermode. It was found that the change in blood flow depended on the extent of the change in hypothalamic temperature and on the ambient temperature. Below 20° C ambient temperature, heating the hypothalamus did not cause vasodilatation and at 30° C ambient temperature, cooling the hypothalamus caused only slight vasoconstriction, but at 25° C ambient temperature, changes in hypothalamic temperature caused changes in blood flow from full vasoconstriction to full vasodilatation.

3. The skin temperature on the trunk was changed by means of water circulated through tubes sewn into a coat worn by the pig. Blood flow in the tail, which was outside the coat, depended on the skin temperature of the trunk, the ambient temperature, and the temperature of the hypothalamus, all of which were varied separately.

4. A thermode was implanted in the epidural space in the cervical region of the spinal cord. The change in blood flow in the tail which accompanied a change in thermode temperature was found to depend on the temperature of the thermode and the ambient temperature. Cooling the spine while the hypothalamus was being heated to 43° C resulted in a decrease in blood flow, but when the spine was heated while the hypothalamus was being cooled the increase in blood flow was only slight.

5. Local stimulus to the tail in the form of infra-red heat, or increased air movement was followed by changes in blood flow even when deep body temperature remained stable.

INTRODUCTION

Within the thermoneutral range of ambient temperature the loss of sensible heat from the body is controlled chiefly by adjustments of tissue insulation brought about by changes in peripheral blood flow. The mechanism by which this flow rate is varied is known to involve temperature receptors not only in the skin but also deep in the body.

The role of the receptors in the hypothalamus on peripheral blood flow has been investigated in several species (Hemingway, Rasmussen, Wikoff & Rasmussen, 1940 in the dog; Ström, 1950 in the cat; Ingram & Whittow, 1962 in the ox; Baldwin & Ingram, 1968 in the pig) and from these studies it may be concluded that, in general, for a given change in hypothalamic temperature blood flow changes by an amount proportioned to the ambient temperature. In studies in which graded changes in hypothalamic temperature have been correlated with simultaneous changes in blood flow in the anaesthetized rabbit (Smith, 1969) and pig (Ingram & Smith, 1970), it was found that blood flow in the carpal region was modified by changes in brain temperature over the whole range between 26 and 42° C. In these experiments, however, values for the rate of blood flow never fell close to zero and for any given brain temperature they were similar at ambient temperatures of both 25 and 35° C. These findings were attributed to the anaesthetic modifying the effects of peripheral stimulation and underlined the need to use unanaesthetized animals.

Blood flow through the extremities may also be influenced by temperature receptors outside the hypothalamus and Simon (1968) has reviewed evidence relating the role of the cervical region of the spinal cord in this respect. Heating or cooling of this region of the cord has been shown by Iriki (1968), and Jessen, Meurer & Simon (1966, 1967) to influence blood flow in the same way that temperature changes in the hypothalamus do.

Blood flow through an extremity is also controlled by the skin temperature on other parts of the body and partly by the skin temperature on the extremity itself (Cooper & Kerslake, 1954; Thauer, 1965).

In the present study, quantitative estimates of the rate of peripheral blood flow have been made in the pig, a species which like man has relatively little external insulation, and in which for this reason the control of tissue insulation may assume greater significance than in some other species. The temperatures of the hypothalamus, the spinal cord and the skin have been subjected to graded changes in an attempt to determine the extent to which stimuli applied in these regions interact with each other.

METHODS

Animals

Fourteen pigs aged between 8 and 14 weeks were used. In seven a thermode was implanted in the preoptic region of the hypothalamus; two had thermodes both in the hypothalamus and in the cervical region of the spinal cord; two had thermistors implanted into the hypothalamus; one control animal had a thermode between the cerebral hemispheres and two controls had thermodes in the thoracic region of the spinal cord.

Thermodes and thermistors

The thermodes and thermistors implanted into the hypothalamus were similar to those previously used in the pig and were implanted using the same technique (Baldwin & Ingram, 1967, 1968; Ingram, 1968). The thermodes which were placed along the spinal cord consisted of a length of vinyl tube 2 mm o.d. Under general anaesthetic and using aseptic precautions a small hole was made in the thoracic region of the vertebral column at the junction of two vertebrae. The tube was doubled and then inserted through the hole to lie over the spinal cord with the tip at C1. A flexible wire pushed down the tube to the bend served both to facilitate the passage along the epidural space, and to make the position of the thermode clear in a radiograph. A blind-ended catheter was then inserted so as to lie part of the way along and close to the thermode and a thermojunction threaded down to the end during experiments, so that temperature changes induced by perfusion of the thermode could be measured. The rate at which the temperature of the thermodes was changed was kept constant (1° C per minute).

Measurement of blood flow rate

The rate of blood flow through the tail was determined by means of venous occlusion plethysmography using a mercury in rubber strain gauge of the type described by Whitney (1953). The gauge was made from a loop of 1 mm o.d. silastic tubing with silver wire contacts at the ends. The wire contacts were mounted in a Perspex holder and the loop passed round the tail and over a peg on the holder. An occlusion cuff 2 cm wide was placed round the base of the tail and inflated rapidly to 75–80 mm Hg during the determination of blood flow rate. The gauge was attached 2 cm posterior to the cuff and the temperature compensator was attached posterior to the gauge.

The gauges were calibrated for resistance changes with changes in length of 8 % of the unstretched length and used on the animals with a constant current of 300 mA. During the determination of blood flow rates the change in voltage across the gauge was displayed on a polygraph recorder and the change in resistance, and hence length, was determined from Ohm's law.

Control of trunk skin temperature by means of a coat

In order to control the skin temperature of the trunk independently of the ambient temperature a specially made coat consisting of a double layer of fine spaced terylene net between which were threaded vinyl tubes 3 mm o.d. at intervals of 1 cm was used on three pigs. The coat which had four holes for the limbs and fastened down the back covered the whole trunk except for an area round the anus and another over the sternum. The neck, head, ears, limbs and tail were not covered. Perfusion of the tubes with warm or cold water changed the temperature of the skin, but the range of temperature which could be achieved was limited to some extent by the

environmental temperature in the controlled room. Changes of skin temperature were made at the rate of 1° C in 5 min. After several sessions of an hour or so the pigs became accustomed to the coat and lay down in the stall, but not until this stage had been reached were measurements of blood flow made.

Skin temperature

The temperature of the skin was measured by means of thermojunctions attached by adhesive tape to the pinna of each ear, both sides of the thorax, and to the tail. *Respiratory frequency* was determined by observation of flank movements.

Experimental procedure

A harness was placed on the pig as soon as it was obtained from the farm and worn continuously from then onwards. Both before and after surgery each pig was lightly restrained in a stall in the temperature-controlled room for several sessions of an hour or more. When the pigs were first introduced into this situation some reassurance from the experimenter was necessary to stop the pig becoming excessively excited and it was found that a warm room and the presentation of food was helpful in this respect. After three or four sessions the animals had usually become accustomed to the environment, were no longer restless and often went to sleep. A period of at least 30 min elapsed after attachment of the thermojunctions, tubes and strain gauge before the experiment began, and measurements were usually taken at intervals of 5 min for the remainder of the experiment.

The experimenters, recording apparatus, and perfusion pumps were all housed outside the temperature-controlled room and the animal observed through a window.

RESULTS

At an ambient temperature of 35° C respiratory frequency increased and at rates above about 50 breaths per minute the associated movement of the body was such that accurate measurements of blood flow could not be made. At 15° C the pigs would not lie still in the stall for long periods and were subject to bouts of shivering with the result that regular determinations of blood flow rate could not be made. At temperatures between 20 and 33° C regular estimations of blood flow could be made over a period of 3–4 hr.

Effect of heating and cooling the hypothalamus

At ambient temperatures between 20 and 33° C, heating the hypothalamus was followed by an increase in blood flow through the tail, and, when there was already a measurable blood flow, cooling was followed by a decrease in the rate. In a series of experiments on five pigs at ambient temperatures of 20, 25 and 30° C and another pig at 23, 27 and 33° C the temperature on the surface of the thermode was held at various values and blood flow determined at 5 min intervals during 30 min periods. Examination of the records (Fig. 1) revealed that a stable blood flow was sometimes not established until 15 min after the change in thermode temperature and for this reason the flow rate at a given thermode temperature was

estimated from the last three readings of each 30 min period. When these blood flow rates at various ambient temperatures were plotted against thermode temperature it was evident that the flow rate was affected by both skin and hypothalamic temperatures (Fig. 2). Some determinations were made after a fall in thermode temperature and some after an increase,

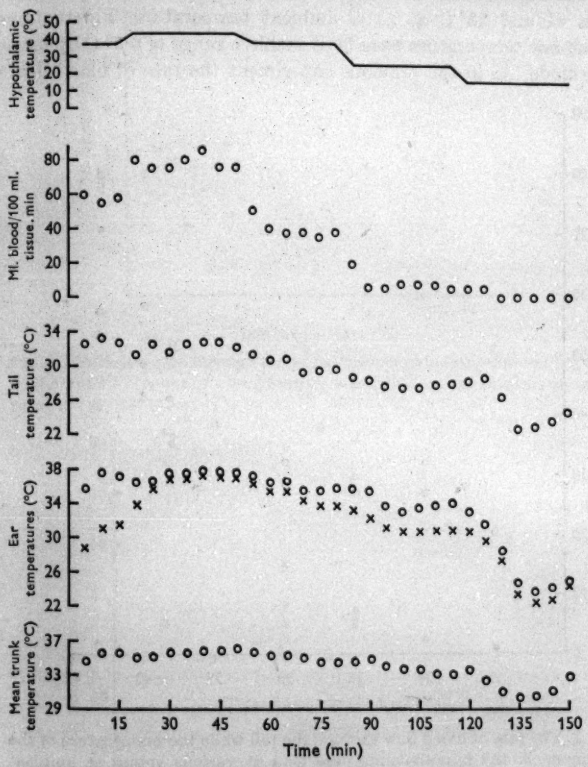

Fig. 1. The effect of changing the temperature of thermode in the hypothalamus on the skin temperature of the ears, the trunk, and the tail as well as on blood flow rate through the tail.

but the direction of the temperature change did not appear to influence the flow rate. At 15° C ambient temperature, if the pig was calm, blood flow was barely detectable at normal hypothalamic temperature and did not increase even when the thermode was heated to 43° C. If the animals

became agitated as happened periodically at this ambient temperature, blood flow to the tail increased.

Effect of heating or cooling the trunk by means of a coat

Skin temperature as determined by thermojunctions on both sides of the thorax was varied between 41 and 33° C at 30° C ambient temperature and between 41 and 29° C at 25° C ambient temperature. Fluctuations in hypothalamic temperature were kept within a range of 0·5° C by means of the thermode. As in the previous experiment the rate of blood flow was

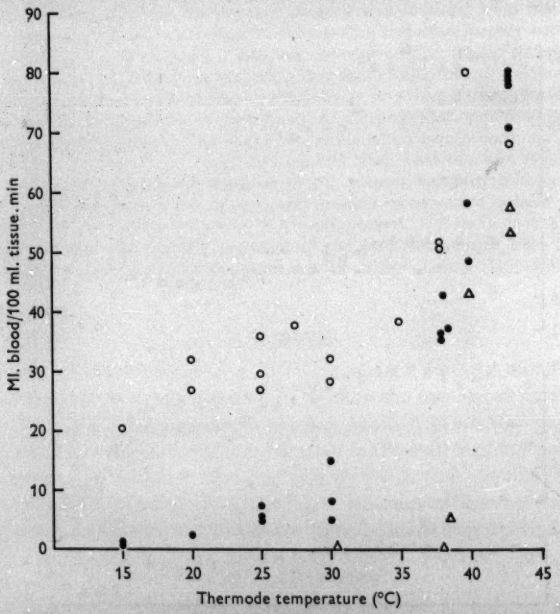

Fig. 2. The rate of blood flow through the tail while the temperature of the thermode in the hypothalamus was held at various values at ambient temperatures of (△) 20° C, (●) 25 °C and (○) 30° C.

estimated from the mean of three readings taken at 5 min intervals at the end of a 30 min period. When flow rate in the tail was plotted against skin temperature on the trunk the slope of the line was similar at both ambient temperatures (Fig. 3), but for any given skin temperature blood flow was greater at the higher ambient temperature. Again the direction of the temperature change did not appear to influence the results.

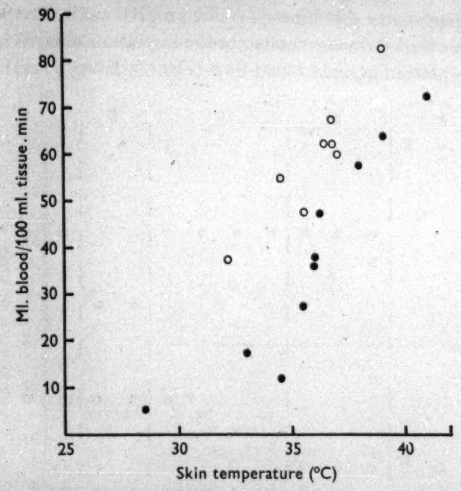

Fig. 3. Blood flow rate through the tail at various skin temperatures imposed on the trunk by means of a temperature-controlled coat at ambient temperatures of (●) 25° C and (○) 30° C.

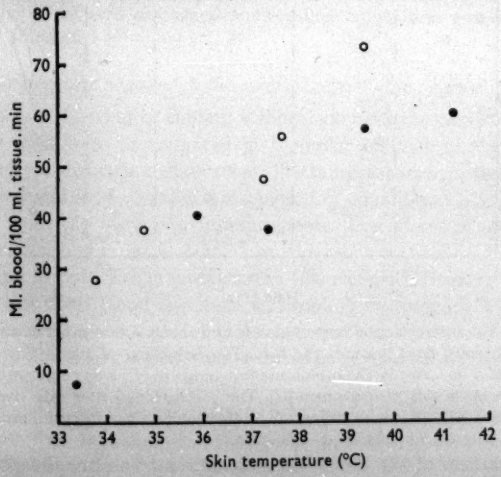

Fig. 4. Blood flow rate through the tail at various skin temperatures imposed by means of a temperature-controlled coat while the thermode in the hypothalamus was (○) 39° C and (●) 35° C. Ambient temperature 30° C.

In other experiments the effects of changing the skin temperature on blood flow were studied during cooling of the hypothalamus. Skin temperature was again plotted against blood flow (Fig. 4). Even a small change in

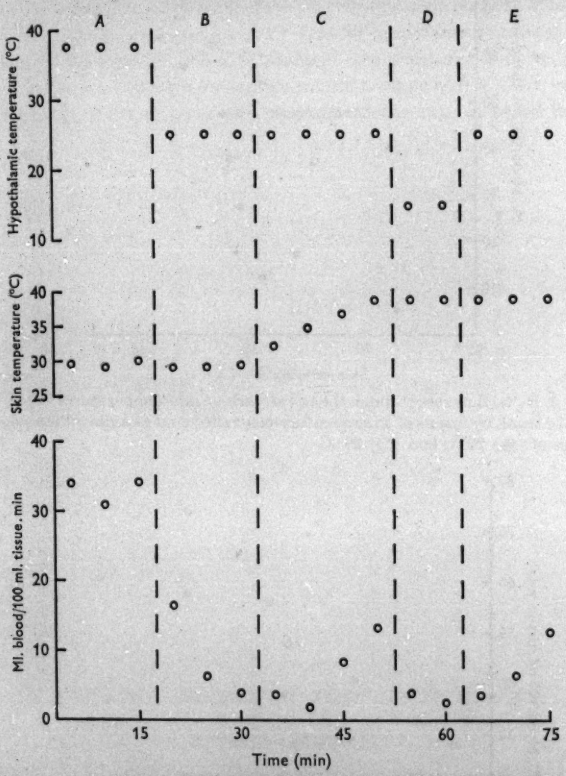

Fig. 5. The interaction of hypothalamic and trunk skin temperature on the rate of blood flow through the tail. The reduction of blood flow which followed a decrease in hypothalamic temperature (*B*) was counteracted by an increase in skin temperature (*C*). Thereafter, blood flow rate decreased again only after further cooling of the thermode in the hypothalamus (*D*).

the temperature of the thermode was sufficient to alter the position but not the slope of the line.

The interaction of skin temperature on the trunk with hypothalamic temperature was further confirmed in experiments in which the hypo-

thalamus was first cooled and when blood flow had been reduced the coat was heated with the result that the flow increased again. Thereafter, further cooling of the hypothalamus was necessary before blood flow could again be reduced (Fig. 5).

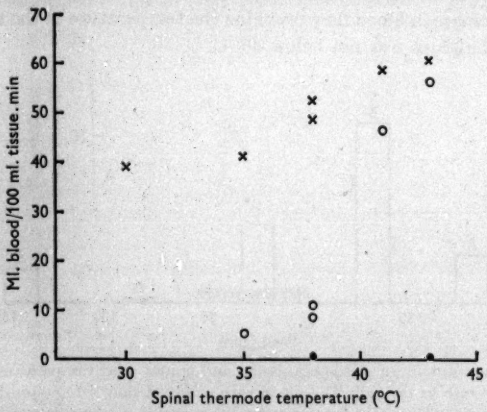

Fig. 6. The effect on blood flow rate in the tail of maintaining the thermode over the spinal cord at various temperatures, at ambient temperatures of (●) 20° C, (○) 25° C and (×) 30° C.

Effect of temperature changes in the cervical region of the spinal cord

The effects of heating or cooling a thermode over the cervical region of the spinal cord were investigated in a similar manner to the effects of changes in hypothalamic temperature. The temperature near the thermode was changed and held constant for a period of 30 min and blood flow rate estimated from the last three measurements. The effects of temperature changes in this region on blood flow in the tail were similar to those recorded when hypothalamic temperature was changed. Heating the spinal thermode increased blood flow rate and cooling decreased it by amounts which depended on the ambient temperatures (Fig. 6). At a low ambient temperature blood flow was not measurable at normal spinal temperatures and did not increase during heating.

In further studies the interaction between hypothalamic and spinal temperature was investigated. When the hypothalamus was warmed, blood flow increased as in previous experiments and it was found that subsequent cooling of the spinal cord decreased the flow rate even though the thermode in the hypothalamus was at 43° C. If the heating in the hypothalamus was now stopped and the cooling of the cord continued, the rate

of blood flow in the tail decreased (Fig. 7). Thus, although cooling the cord while the hypothalamus was warm reduced blood flow, the effect of cooling the cord did not completely override the effect of heating the hypothalamus. When the hypothalamus was cooled and blood flow decreased, additional warming of the spinal cord to 43° C was accompanied by a small increase in blood flow provided the temperature of the thermode in the hypothalamus was not below 35° C.

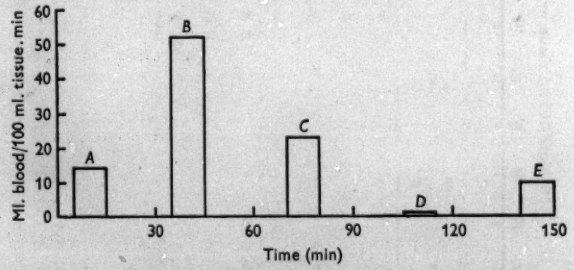

Fig. 7. The interaction of hypothalamic and spinal cord temperatures on blood flow rate in the tail. The mean rate of blood flow after equilibrium had been reached is given for various combinations of temperature in the two thermodes: *A*, hypothalamus 39° C, spine 39° C; *B*, hypothalamus 43° C, spine 39° C; *C*, hypothalamus 43° C, spine 20° C; *D*, hypothalamus 39° C, spine 20° C; *E*, hypothalamus 39° C, spine 39° C.

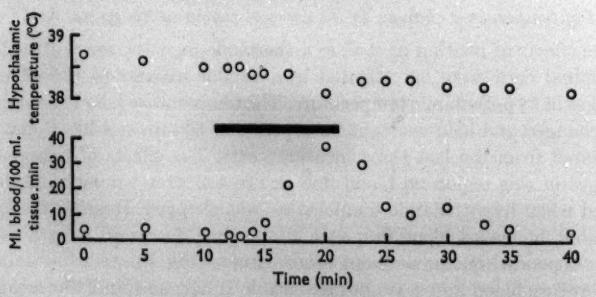

Fig. 8. The effect of blood flow rate through the tail of infra-red heat directly on the tail. Bar indicates infra-red on.

Effect of local thermal stimulation of the tail

In pigs with a thermistor implanted into the preoptic region of the hypothalamus blood flow changes were induced by application of infra-red heat, or by changes in air movement. When the tail was irradiated with

infra-red heat, while the body was shaded, vasodilatation took place rapidly (Fig. 8), and blood flow returned to its previous level when the heater was turned off. These changes in blood flow did not appear to be related to changes in hypothalamic temperature. Irradiation of the body while the tail was shaded also caused vasodilatation, but if body temperature also increased it did so after blood flow rate had begun to increase.

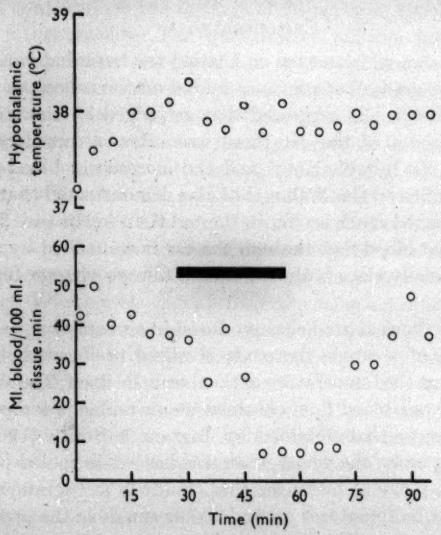

Fig. 9. The effect on blood flow rate through the tail of a fan blowing over the tail. Bar indicates fan on.

Turning on a fan which blew air over the tail was followed by vasoconstriction, and when the fan was turned off blood flow returned to its previous value again. Again these changes in blood flow were not correlated with changes in hypothalamic temperature (Fig. 9).

Skin temperatures

As may be seen (Figs. 1 and 7) the skin temperature on the tail was a fair indicator of blood flow at certain ambient temperatures, but the correlation with flow rate was less obvious when the ambient temperature was high or low.

Controls

Changing the temperature of the thermodes placed between the cerebral hemispheres or in the thoracic region of the spinal cord had no effect on the rate of blood flow in the tail.

DISCUSSION

The vasodilatation which occurs on heating the hypothalamus has been reported in many species but previous studies on the conscious pig (Baldwin & Ingram, 1968), had suggested that as judged by the temperature changes on the pinna of the ear there was often a considerable delay between heating the hypothalamus and the increase in blood flow. The present study confirmed this finding, but also demonstrated that the blood flow changes occurred much sooner in the tail than in the ear. This raises the possibility that blood flow through the ear is controlled by a separate mechanism, especially since both ears do not always change temperature at the same time.

Because of the thermal gradients produced in surrounding tissues when the temperature of a single thermode is raised or lowered, blood flow was plotted against the temperature of the thermode itself. The shape of the curve describing tail blood flow obtained at an ambient temperature of 25° C was similar to that obtained by Ingram & Smith (1970) on the anaesthetized pig when the whole brain was heated or cooled between 36 and 42° C. From previous measurements (Baldwin & Ingram, 1968) it is probable that the temperatures around the thermode in the present study were also in this range. However, in the present experiment, at 25° C ambient temperature, tail blood flow fell to near zero when the hypothalamus was cooled, while in the anaesthetized pigs used by Ingram & Smith (1970) complete vasoconstriction was not seen. The explanation for these differences may be that the degree of vasoconstriction which can be elicited by cooling the hypothalamus is limited in the absence of normal peripheral stimulation, for in the anaesthetized pig changes in ambient temperature did not influence blood flow. By contrast in the present study on conscious pigs, blood flow clearly depended on both peripheral and central stimuli. In general, cooling the hypothalamus had rather less effect than heating on blood flow, and this might be expected if the change in the radius of the blood vessels was related linearly to the temperature change at the receptors, since flow is related to r^4 rather than to r. In the present study cooling the hypothalamus was, however, always followed by a decline in blood flow in contrast with Brendel's (1960) observation that in the dog peripheral resistance as judged by blood pressure was little

affected when the whole brain was cooled, while trunk temperature was held constant; Ström (1950) also reported that cooling the hypothalamus of the anaesthetized cat failed to cause vasoconstriction at normal rectal temperature. Again these failures to elicit peripheral vasoconstrictions could be attributed to the effects of the anaesthetic.

The vasoconstriction after cooling and the vasodilatation after heating the spinal cord confirm the findings of Jessen *et al.* (1966, 1967) and Iriki (1968) in other species. The quantitative relation between the spinal thermode temperature and blood flow in the tail and the interaction with ambient temperature were similar to those for changes in the temperature of the hypothalamus. What needs to be determined is whether this similarity stems from the effects of heating the spine being mediated via the hypothalamus, or whether the effects are produced directly. It has been established by Kosaka, Simon, Thauer & Walther (1969) that with respect to panting the influence of thermal stimuli applied to the spine is mediated by structures in the brain rather than directly, but the possibility remains that the effects of spinal temperature on blood flow may be, at least in part, direct. Whether the signals from the spine act directly or indirectly, it would appear that neither the spinal nor the hypothalamic receptors override the other but that the final signal to the blood vessels is determined by the integration of information from both sets of receptors.

The blood flow in the tail is also modified by local changes in the temperature on the skin of the trunk, as well as by direct thermal stimulus to the tail itself and similar effects have been shown elsewhere (Kerslake & Cooper, 1950; Cooper & Kerslake, 1953, 1955). The effect of changing the trunk skin temperature is influenced by the deep body temperature and the plot of skin temperature against blood flow for different body temperatures resembles those published for man (Wyndham, 1965).

The rate of blood flow through the tail may thus be influenced by changes in the temperature of the tail itself, the skin of the trunk, the cervical region of the spinal cord, and the preoptic region of the hypothalamus. The actual flow rate at any given time is determined by the integration of signals from all these and possibly other receptors, but whether the origin of the signals is peripheral or central appears to be relatively unimportant, with respect to the output signal to the control system. Probably the simplest explanation is to suppose that nervous impulses from receptors at a number of sites impinge on the same interneurone. The output of this interneurone which would depend on the balance of input signals might then be arranged to attenuate a particular thermoregulatory mechanism. Some evidence to support this idea derives from the studies of Wit & Wang (1968) and Hellon (1970*a*, *b*) who found some neurones in the hypothalamus which were sensitive to local changes in temperature but not

to ambient temperature and others which were sensitive to both local temperature and the temperature at the periphery.

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With 10 *text-figures*
Printed in Great Britain

ACTIVATION OF TRANSMITTER RELEASE BY STRONTIUM AND CALCIUM IONS AT THE NEUROMUSCULAR JUNCTION

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(*Received 30 October 1970*)

SUMMARY

1. The interaction between Ca and Sr ions on quantal transmitter release at the frog neuromuscular junction was studied, using conventional electrophysiological techniques.

2. While Ca ions always activate transmitter release, the activating action of Sr ions depends on the Ca ion concentration in the medium; at low [Ca], strontium ions enhance the release, but at higher [Ca] they inhibit it. It is postulated that there is a [Ca] at which Sr ions do not affect transmitter liberation.

3. When Sr activates release, its effect and the effect of Ca add in a more than linear fashion.

4. Magnesium ions inhibit the release induced by Sr.

5. The results can be explained by assuming that Ca and Sr act on the same site, at some stage of the process of quantal transmitter release. The affinity of both ions towards the sites is approximately the same, but the effectiveness of Sr is much smaller.

INTRODUCTION

Calcium ions play an important role in the coupling between nerve terminal depolarization and acetylcholine release at the neuromuscular junction. In the absence of Ca ions the nerve action potential invades the terminals, but fails to induce liberation of transmitter (Katz & Miledi, 1965). It has been found recently (Miledi, 1966; Dodge, Miledi & Rahamimoff, 1969) that Sr ions are able to replace Ca ions in this respect (see also Mines, 1911). When Sr ions are substituted for Ca, the release of transmitter has similar properties to the normal release under calcium; it is liberated spontaneously and after nerve stimulation; the release is quantal in nature; when the mean number of quanta liberated per nerve impulse

(quantal content, m) is low, typical fluctuations in the end-plate potential (e.p.p.) amplitude appear, which are predicted accurately by the Poisson theorem (for review on normal properties of release, see Katz, 1969).

Though the activation of the release process by Ca and Sr seems to be qualitatively quite similar, there is an important quantitative difference in the action of these two ions. Under normal Ca concentration of 1·8 mM, the mean quantal content is several hundred (Katz, 1962). With similar strontium concentrations, the mean quantal content is only a few quanta.

There are several questions that arise regarding the action of Ca and Sr. First, are Sr and Ca enabling transmitter to be liberated by the same release mechanism? If so, what is the basis of the difference in potency? Is the shape of the relation between divalent ion concentration and release similar for both Ca and Sr? What is the effect of competitive inhibitors to Ca, such as Mg (see Jenkinson, 1957), on the release by Sr? Is it possible to employ the difference between Ca and Sr for further understanding of the release process? It is the purpose of this work to examine several of these questions.

METHODS

The experiments were performed on the sartorius nerve-muscle preparation of the frog *Rana ridibunda*, at room temperature of 19–24° C.

During the dissection and the mounting of the preparation, it was immersed in Ringer solution (standard composition: 116·0 mM-NaCl, 2·0 mM-KCl and 1·8 mM-$CaCl_2$). Thereafter, the divalent ion composition of the medium was varied by isotonic substitution for Na. It is unlikely that the variation in $[Na]_o$ has a significant effect on the transmitter release properties of the nerve endings under the present circumstances (e.g. see Kelly, 1965; Rahamimoff & Colomo, 1967). Two restrictions were imposed on the degree of variation of the divalent ion concentration. First, in most of the experiments, the total divalent ion concentration was never lowered below an arbitrary level of 1 mM in order to avoid large changes in the excitability of nerve (e.g. see Frankenhaeuser, 1957). Secondly, total divalent ion concentration was always adjusted in such a way as to keep the end-plate potential subthreshold for muscle excitation.

Special precautions were taken to avoid large artifacts when using Sr, due to Ca contamination. After the initial wash of the preparation and the bath with Ca-free medium, there is still a considerable amount of Ca in the muscle, most of it presumably in the sarcoplasmic reticulum (e.g. see Ebashi & Endo, 1968). This Ca leaks out during the course of the experiment. The amount of Ca liberated in the presence of Sr was estimated by Miss E. Eilander.

Frog sartorii were dissected and mounted on a platinum frame. They were incubated for 16 hr in a normal Ringer solution containing radioactive ^{45}Ca, at 4° C. Continuous bubbling of air through the incubation chamber was employed for mixing. After this period, the chamber and the muscle were transferred to room temperature and allowed to equilibrate. Thereafter, the muscle with the frame were transferred to successive test-tubes at 5–10 min intervals. The bubbling of air continued throughout the experiment. The washout of Ca was thus determined for 3–3½ hr. At the end of the experiment the muscle was dissolved in concentrated nitric acid (2 M-HNO_3). The radioactivity was determined by a liquid scintillation counter

(Tricarb-Packard) in the washout fluid and in the muscle. Most of the ^{45}Ca was released during the first hour. This presumably reflects mainly the diffusion from the extracellular space. Thereafter the contribution of the muscle to the bath [Ca] was relatively small. If one assumes that the specific activity of the released Ca is similar to that in the incubation mixture, the amount of Ca released to the bath is approximately 5.10^{-8} moles for a 30 min period. After the first hour, this amount will increase the [Ca] in a 5 ml. bath by approximately 10 μM.

For electrophysiological measurements only one of the surfaces of the muscle is fully exposed to the external medium, while in the washout tubes, both surfaces were in direct contact with the outside. Furthermore, during the washout continuous bubbling of air produced the necessary stirring, while during the electrical recording, the external medium was unstirred. To minimize the effects of the Ca efflux, frequent intermediate washings of the preparation were made. Initially, Ca was washed out by flowing several hundred ml. of a medium with low [Ca], during 90–120 min, through the preparation chamber of 5 ml. capacity. Subsequently, recordings were never taken for periods longer than 20 min without an intermediate exchange of the bathing medium. Even with these precautions, there is probably still a contamination with Ca of approximately 20–80 μM. However, such contamination does not have a noticeable influence on quantal release.

E.p.p.s were elicited at a rate of 0·1–0·25/sec in the presence of strontium and 0·2–0·5/sec in the presence of calcium. These relatively low rates of nerve stimulation were found necessary in order to avoid progressive accumulation of small after-effects (N. Moran & R. Rahamimoff, unpublished observations).

End-plate responses were recorded intracellularly with conventional 3·0 M-KCl-filled micropipettes. In the range of divalent ions concentrations used here, the e.p.p. amplitude fluctuated in accordance with the quantal release hypothesis (del Castillo & Katz, 1954*b*). Therefore a large number of e.p.p.s was used to estimate the quantal content by the three available methods (see del Castillo & Katz, 1954*b*; Martin, 1966). When the quantal content was estimated by the coefficient of variation method, a correction for non-stationarity was frequently used (Colomo & Rahamimoff, 1968). E.p.p. amplitude was usually averaged automatically by a computer for averaging transients (C.A.T. 1000, TMC).

RESULTS

Non-linear addition of the effects of Sr and Ca

The first question examined was whether Ca and Sr induce transmitter liberation by the same mechanism. If Ca and Sr ions are activating the nerve-evoked release of transmitter by two separate non-interacting processes, then one would predict that the combined effect of Sr and Ca is, at most, the algebraic sum of the individual effects. The combined post-synaptic effect might be of course less than the algebraic sum of the individual effects, due to effects of divalent ions on the post-synaptic sensitivity (del Castillo & Engbaek, 1954; Nastuk & Liu, 1966), and due to non-linear summation (del Castillo & Katz, 1954*b*; Martin, 1955). If, on the other hand, the two ions are operating on the same mechanism, then in a certain range of concentrations, the presence of one of the ions might facilitate the release by the other, and vice versa. Fig. 1 shows that the

combined action of Ca and Sr is not equal to, or less than, the sum of their separate actions, but is larger; in the presence of 0·3 mM-Ca and no Sr the averaged e.p.p. amplitude (R_{Ca}) is 0·117 mV; in the presence of 2·0 mM-Sr and no Ca the amplitude (R_{Sr}) is 0·119 mV; when the two divalent ions are present simultaneously, each at the same concentration as before, the e.p.p. amplitude ($R_{Ca, Sr}$) is 0·355 mV. This experiment illustrates that in a certain range of Ca and Sr concentration, their effects add in a more-than-linear way. The ratio $u = (R_{Ca, Sr})/(R_{Ca} + R_{Sr})$ is in this case 1·50. The ratio u was estimated in seven additional experiments and found to be in the range 1·20–3·33 with a mean value of 1·66.

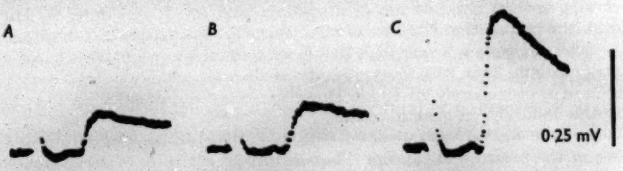

Fig. 1. Non-linear summation of the effects of Ca and Sr ions on transmitter release at the frog neuromuscular junction. *A*, Average response of 0·117 mV to 270 nerve stimuli. Mean quantal content of 0·61. Bathing medium contains 0·3 mM-Ca. *B*, average response of 0·119 mV to 135 nerve stimuli. Mean quantal content of 0·72. Bathing medium contains 2·0 mM-Sr. *C*, average response of 0·355 mV to 135 nerve stimuli. Mean quantal content 2·08. Bathing medium contains 0·3 mM-Ca *and* 2·0 mM-Sr.

Note that when the two activating divalent ions were present simultaneously, transmitter release is 56% greater than the sum of the individual releases ($u = 2{\cdot}08/[0{\cdot}61 + 0{\cdot}72] = 1{\cdot}56$). 1 mM-Mg was present throughout the experiment. Voltage calibration of 0·25 mV applies to all three records. Averaging step 125 μsec. Stimulation rate of 0·5/sec in *A* and 0·25/sec in *B* and *C*.

The more-than-linear addition of the effects of Ca and Sr can be due either to an increase in the size of the quantum or to an increase in the quantal content. Analysis of the quantal contents of the above-mentioned experiments shows that the latter factor is predominant. For example in the experiment where the value $u = 3{\cdot}33$ was obtained, the quantal contents were $0{\cdot}92 \pm 0{\cdot}06$ (S.E. of mean) for Ca alone (m_{Ca}); $1{\cdot}39 \pm 0{\cdot}03$ (S.E. of mean) for Sr alone (m_{Sr}) and $7{\cdot}7 \pm 0{\cdot}09$ (S.E. of mean) for the combined action of Ca and Sr ($m_{Ca, Sr}$). The ratio $m_{Ca, Sr}/(m_{Ca} + m_{Sr}) = 3{\cdot}33$ shows that the origin of this *more-than-linear addition* is presynaptic and comes from an increase in the number of quanta released by the nerve impulse. These results also suggest that Ca and Sr act on some common mechanism in the release process. One can describe formally the quantitative dependence of transmitter liberation on [Ca] as a reaction between Ca

ions and a specific site X on the nerve terminal (del Castillo & Katz, 1954*a*; Jenkinson, 1957; Dodge & Rahamimoff, 1967):

$$Ca + X \underset{}{\overset{k_1}{\rightleftharpoons}} CaX, \tag{1}$$

where K_1 is the dissociation constant. For release to occur at least four CaX (or Ca ions) have to co-operate. Mg ions compete with Ca for X, giving

$$Mg + X \overset{k_2}{\rightleftharpoons} MgX, \tag{2}$$

where K_2 is the dissociation constant for MgX.

The following equation, which is based on this co-operative assumption, describes the dependence of release on [Ca]:

$$m = K\left(\frac{[Ca]/K_1}{1 + [Ca]/K_1 + [Mg]/K_2}\right)^4, \tag{3}$$

where K is a constant.

Since it seems that Sr ions are acting on the same sites as Ca, one may assume that

$$Sr + X \overset{k_3}{\rightleftharpoons} SrX, \tag{4}$$

where K_3 is the dissociation constant for SrX.

The inhibitory effect of Sr ions on transmitter liberation

If we continue with the formal description of the release properties, we may ask the following question. Why are Sr ions less effective than Ca ions? The difference in potency is obvious in Fig. 1. To obtain approximately the same response, 2·0 mM-Sr are needed, but only 0·3 mM-Ca. Such a situation may be either due to lower affinity of Sr to the site X, (i.e. a high dissociation constant of Sr) or due to lower effectiveness of the SrX complex. Of course both possibilities may co-exist. If the difference in potency between Sr and Ca is only due to lower affinity of Sr to X, addition of Sr, when the release is already substantially activated by Ca, will lead to a small increase in the quantal release. On the other hand, if the difference is due to lower effectiveness of SrX, then addition of Sr will reduce the fraction of sites occupied by Ca and will reduce the quantal output. Fig. 2 shows that indeed the effectiveness of SrX is lower; addition of Sr decreases the e.p.p. amplitude and the quantal content. In the presence of 1·8 mM-Ca, $\bar{R}$ is 4·44 mV and m, calculated by the coefficient of variation method, is 307. Addition of 2·0 mM-Sr reduces the $\bar{R}$ to 2·64 mV and the quantal content to 156. This effect is concentration dependent. When the concentration of Sr was progressively increased, the e.p.p. amplitude decreased accordingly, as illustrated in Fig. 3.

The relation between [Sr] and transmitter release

The analysis of the dependence of transmitter release at the frog neuromuscular junction on the extracellular Ca concentration, revealed that for a liberation of a quantum of transmitter a co-operative action of at least 4 CaX (or four Ca ions) is necessary. The value of four was obtained by estimating the initial slope of the log Ca–log release relation. At low [Ca]

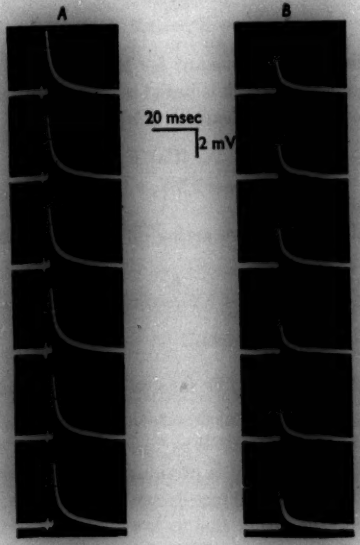

Fig. 2. Inhibition of transmitter release by Sr ions. The neuromuscular preparation was bathed in medium containing 1·8 mM-Ca and (+)-tubocurarine, 10^{-6} g/ml., throughout the experiment. *A*, Control with no Sr ions present. Average amplitude of e.p.p. = 4·44 mV. Quantal content calculated by the coefficient of variation method = 307 (number of responses (N) = 190). *B*, Addition of 2·0 mM-Sr reduced the average amplitude of e.p.p. to 2·64 mV and the quantal content to 156 (N = 190). Time calibration of 20 msec and voltage calibration of 2·0 mV apply to both sets of records.

the denominator of eqn. (3) can be considered approximately constant with small changes in [Ca] and therefore the slope of the log–log plot yields an estimate of the power of the relation (Dodge & Rahamimoff, 1967). It was found impossible to perform the same analysis for Sr; the average release of transmitter in the 10^{-4} M range of [Sr] is so low, that one needs many

thousands of responses to obtain reliable measures of the quantal content for every single Sr concentration. Since the optimal rate of stimulation cannot exceed 0·2–0·3/sec, in order to avoid accumulation of after effects (N. Moran & R. Rahamimoff, unpublished), it would have been necessary to spend several hours on each point, which is not feasible in practice. Therefore an alternative, though less satisfactory, procedure was employed. It

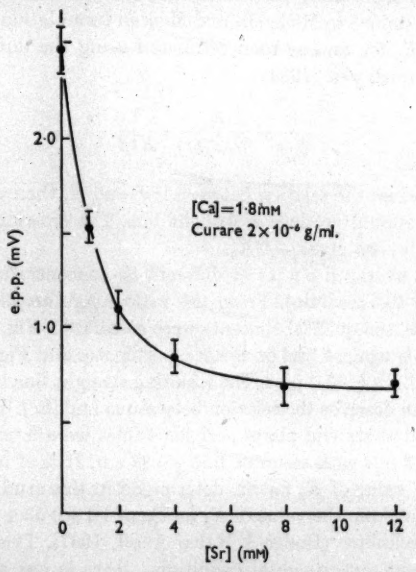

Fig. 3. Inhibition of e.p.p. amplitude by Sr ions. 1·8 mM-Ca and (+)-tubocurarine, $2 \cdot 10^{-6}$ g/ml., present throughout the experiment. Each point is an average response to sixty stimuli. Stimulation rate 0·25/sec. Vertical bars denote ± 1·0 S.E. of mean.

was assumed that since Ca and Sr are acting presumably on the same sites similar kinetics apply to both ions. Hence, the relations found for Ca (Dodge & Rahamimoff, 1967) were tested for Sr. An additional constant β was introduced to denote the relative effectiveness of SrX, so, if $\beta = 0 \cdot 5$, the effectiveness of SrX is one-half of that of Ca. Therefore the relation between quantal content and [Sr] is assumed to be

$$m = K\left(\frac{\beta[\mathrm{Sr}]/K_3}{1+[\mathrm{Sr}]/K_3}\right)^4. \qquad (5)$$

When both Ca and Sr are present, the relation between the divalent ion concentration and the release will be given by

$$m = K\left(\frac{[\mathrm{Ca}]/K_1 + \beta[\mathrm{Sr}]/K_3}{1 + [\mathrm{Ca}]/K_1 + [\mathrm{Sr}]/K_3}\right)^4, \tag{6}$$

(see also Discussion).

Estimation of K_3. Taking the fourth root of both sides of eqn. (5), one obtains a form similar to Michaelis and Menten formulation. The dissociation constant K_3 for Sr was then estimated using the double reciprocal Lineweaver & Burk plot (1934):

$$\frac{1}{\sqrt[4]{m}} = \frac{K_3}{K^{\frac{1}{4}}\beta[\mathrm{Sr}]} + \frac{1}{K^{\frac{1}{4}}\beta}. \tag{7}$$

If eqn. (5) describes the relation between [Sr] and m, then a plot of $1/\sqrt[4]{m}$ against $1/[\mathrm{Sr}]$, should produce a straight line. The crossing point of this line with the abscissa gives $-1/K_3$.

Fig. 4 shows averaged e.p.p.s at different Sr concentration (no Ca ions were added to the medium). From the ratio *e.p.p. amplitude/miniature e.p.p. amplitude*, the quantal contents were calculated. Fig. 5*A* illustrates the relation of m against [Sr] on linear co-ordinates and Fig. 5*B* gives the Lineweaver & Burk (1934) plot. The resulting straight line is an indication that eqn. (5) can describe the relation between m and [Sr]. The value of K_3 was determined at six end-plates and the values were found to be in the range of 1·0–2·2 mM with mean of $1{\cdot}55 \pm 0{\cdot}38$ S.D. It is of interest to note that the mean value of K_3 for Sr, determined in this study, is not significantly different from the value of K_1 for Ca of $1{\cdot}1 \pm 0{\cdot}53$ S.D., determined by the same technique (Dodge & Rahamimoff, 1967). This suggests that under the present experimental conditions, there is not a large affinity difference between Ca and Sr toward the postulated sites X.

It is impossible in this set of experiments to distinguish between K and β in eqn. (5). However, the product $K^{\frac{1}{4}}\beta$ can be estimated from eqn. (7) and Fig. 5*B*. Having the value of $K^{\frac{1}{4}}\beta$, one can plot eqn. (5) over a wide range of Sr concentrations. The continuous line in Fig. 5*A* represents the equation

$$m = \left(\frac{2{\cdot}4[\mathrm{Sr}]/K_3}{1 + [\mathrm{Sr}]/K_3}\right)^4,$$

on linear co-ordinates. There is a reasonable fit between the experimental results and the theoretical line.

Estimation of β. Figs. 3 and 6 show that the effect of Sr on transmitter release depends on the extracellular Ca concentration. At low [Ca], addition of Sr increases the quantal output (Fig. 6) while at high [Ca] the same

concentration of Sr diminishes it. These findings were interpreted as a lower effectiveness of SrX compared to CaX in the process of transmitter liberation. The effectiveness term β cannot be determined directly from eqn. (5), but it can be estimated employing the first derivative of the

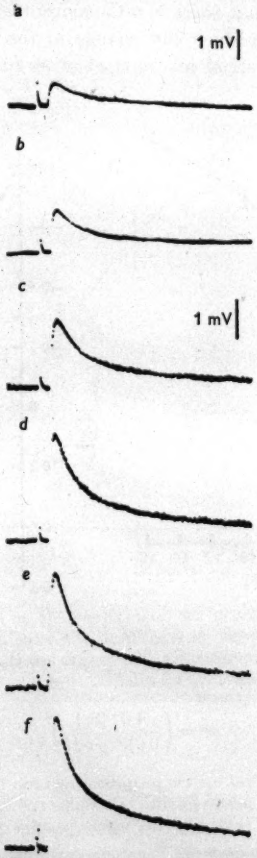

Fig. 4. The dependence of e.p.p. amplitude on Sr ion concentration. Each record is an average of sixty-six responses. Strontium concentrations are: a = 2·0 mM; b = 3·0 mM; c = 4·0 mM; d = 6·0 mM; e = 8·0 mM; f = 10·0 mM. Stimulation rate 0·16/sec. Averaging step 62·5 μsec. Upper calibration applied to a and b; lower calibration applied to c–f.

quantal content in eqn. (6) in respect of [Sr]. Solving $dm/dSr = 0$, one obtains

$$\beta = \frac{Ca'/K_1}{1+Ca'/K_1} = \frac{Ca'}{K_1+Ca'}. \tag{8}$$

This solution means that there is a Ca concentration-Ca', where addition of Sr ions does not produce any change in the quantal output. Fig. 7 illustrates three theoretical relations of m as function of [Sr], at three

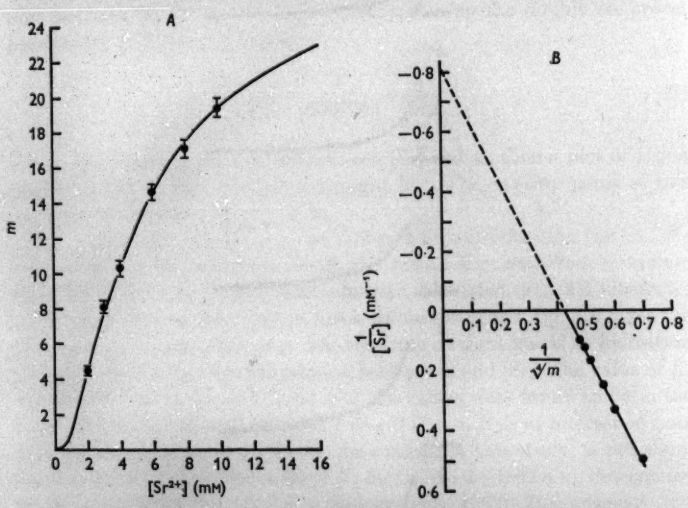

Fig. 5. The dependence of the quantal content on strontium ion concentration. The points were derived from the experimental results shown in Fig. 4. *A*, Linear co-ordinates. The points are the experimental results and the line is the theoretical relation

$$m = \left(\frac{1{\cdot}97\,[Sr]}{1+[Sr]/1{\cdot}22}\right)^4.$$

B. The same as *A* on double reciprocal relation (Lineweaver & Burk's plot (1934). From the intercept with the x-axis the value of $K_3 = 1{\cdot}22$ was estimated. From the slope and the intercept, the other constants of the equation were found.

different [Ca]([Ca] > Ca'; [Ca] = Ca'; [Ca] < Ca'). The upper curve corresponds experimentally to a situation similar to Fig. 3 and the lower curve to Fig. 6. In several experiments Ca' was estimated by interpolation and found to be 0·5–0·7 mM, giving values of β between 0·3 and 0·5. There-

fore the maximal release in Sr is $(0{\cdot}3)^4$ to $(0{\cdot}5)^4$ of the maximal release in Ca. In other words, at same molar concentrations the expected release in Sr is only 0·81–6·25 % of the corresponding release in Ca. Such ratios were indeed found in practice.

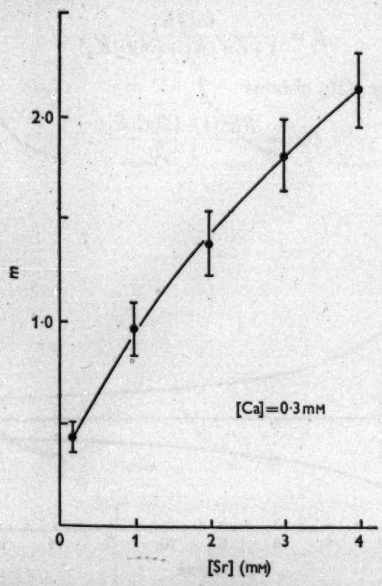

Fig. 6. The relation between [Sr] and transmitter release in the presence of Ca = 0·3 mM. Quantal content was estimated by the 'failure and success' method

$$m = \ln\left(\frac{\text{Number of stimuli}}{\text{Number of failures}}\right).$$

Vertical bars denote ± 1·0 S.E. of mean.

The effect of Mg ions on Sr-induced release

The above-mentioned experiments suggest that Ca and Sr exert their action by acting on the same postulated sites on the presynaptic nerve terminal. It is expected, therefore, that competitive inhibitors of Ca should inhibit also the Sr-induced release. Such an inhibitor is Mg (Jenkinson, 1957); and indeed addition of Mg decreases the mean amplitude of the e.p.p. and the mean quantal content (Fig. 8*A–F*).

The peculiar way of the interaction between Ca and Sr permits a further

prediction. The form of eqns. (6) and (8) for the case where Mg ions are also present, should be respectively

$$m = K\left(\frac{[\mathrm{Ca}]/K_1+\beta[\mathrm{Sr}]/K_3}{1+[\mathrm{Ca}]/K_1+[\mathrm{Mg}]/K_2+[\mathrm{Sr}]/K_3}\right)^4, \tag{9}$$

and

$$\beta = \frac{Ca'/K_1}{1+Ca'/K_1+[\mathrm{Mg}]/K_2}, \tag{10}$$

or rearranging eqn. (10) obtains

$$Ca' = \frac{\beta K_1(1+[\mathrm{Mg}]/K_2)}{1-\beta}. \tag{11}$$

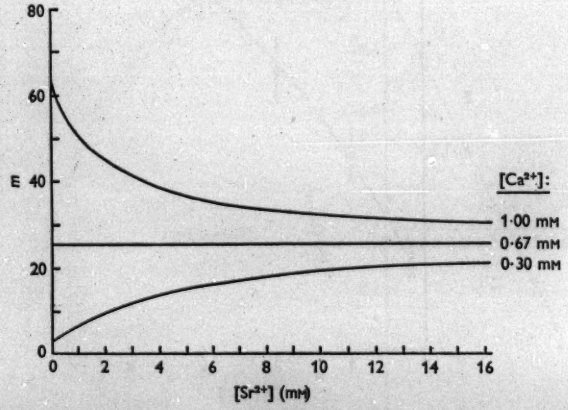

Fig. 7. Theoretical relation between [Sr] and quantal content at three different extracellular [Ca] as shown in Figure. The relations are according to the equation

$$m = K\left(\frac{\beta[\mathrm{Sr}]/K_3+[\mathrm{Ca}]/K_1}{1+[\mathrm{Ca}]/K_1+[\mathrm{Sr}]/K_3}\right)^4,$$

where $K = 1000$; $K_3 = 1{\cdot}5$; $K_1 = 1{\cdot}0$; $\beta = 0{\cdot}4$. Compare Fig. 3 with Fig. 6 and see text.

From eqn. (11) it follows that Ca' depends on the magnesium concentration in the medium. The higher the [Mg], the higher will be Ca'. This predicted property of the system suggested the following experiment: the averaged e.p.p. amplitude and quantal content were estimated at 1·0 mM-Ca (Fig. 9*A*). Addition of Sr (2·0 mM) as expected reduced $\bar{R}$ and m (Fig. 9*B*). Now the composition of the medium was changed to contain 1·0 mM-Ca and 4·0 mM-Mg (Fig. 9*C*). Since the competitive inhibitor Mg is present, mean R and m were smaller than in Fig. 9*A*. The addition of

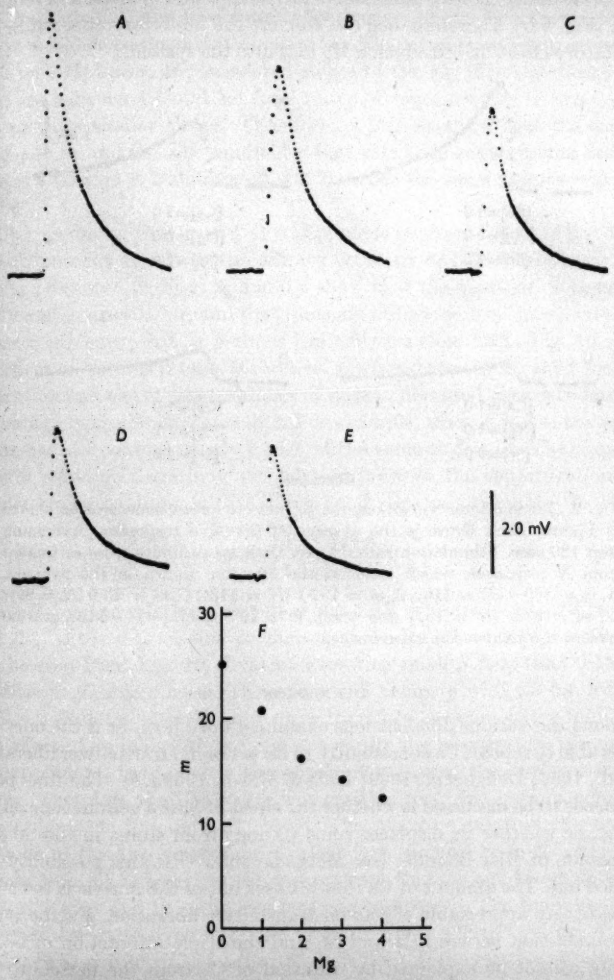

Fig. 8. Inhibitory effect of Mg ions on transmitter release. 4·0 mM-Sr present throughout the experiment. Average responses to 120 stimuli; *A*, Mg = 0·0 mM; *B*, Mg = 1·0 mM; *C*, Mg = 2·0 mM; *D*, Mg = 3·0 mM; *E*, Mg = 4·0 mM. Averaging step 250 μsec. Voltage calibration = 2·0 mV. Stimulation rate 0·22/sec. *F*, Quantal content as function of [Mg]. The points were derived from the experimental results in *A*–*E*.

2·0 mM-Sr under these circumstances *increased* $\bar{R}$ and m instead of reducing them (Fig. 9*D*). Therefore, one can convert the inhibitory effect of Sr to a facilitatory effect by introducing Mg ions into the system.

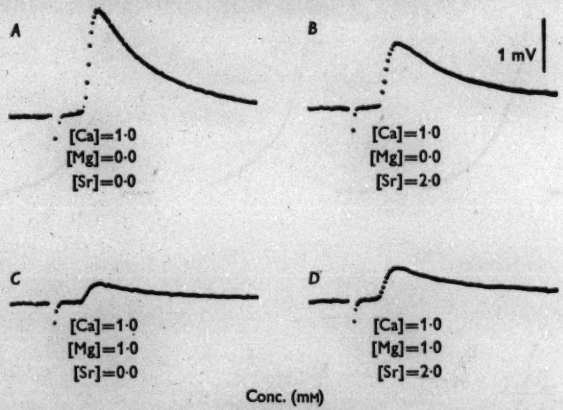

Fig. 9. Triple interaction among Ca, Sr and Mg ions. Concentration shown in Figure. Each figure is the average of fifty-five responses. Averaging step 125 μsec. Stimulation rate 0·1/sec. Quantal contents were estimated from N responses, which included the fifty-five shown in the average. *A*, m = 150·6 (N = 119); *B*, m = 107·1 (N = 113); *C*, m = 25·9 (N = 90); *D*, m = 33·3 (N = 107) (see text). $0{\cdot}7 \times 10^{-6}$ g/ml (+) −tubocurarine present throughout the experiment.

DISCUSSION

Among the various divalent ions examined until now, Sr is the only one that is able to replace Ca consistently in its action on transmitter liberation (Miledi, 1966; Dodge *et al.* 1969; Katz & Miledi, 1969*a*, *b*). The first point that needs to be discussed is whether the effect of Sr is a genuine one on the release, or whether Sr displaces some Ca ions from stores in the muscle. The results of Miss Eilander (see Methods) show that this possibility can be ruled out. The amount of Ca that diffuses out of the muscle is too small to create any appreciable effects on transmitter liberation. Furthermore, the competition between Ca and Sr, and the triple interaction of Ca, Sr and Mg cannot be explained by diffusion of Ca from the muscle to the extracellular space. Therefore, it can be concluded that Sr ions have a direct effect on quantal transmitter release.

The mode of action of Sr is quite similar to that of Ca. Both ions act presumably on the same presynaptic sites. The main difference in their influence is quantitative: Sr is less potent than Ca. This difference in

potency can formally be due to a lower affinity of Sr for the postulated sites X (high K_3), due to a lower effectiveness of the SrX complexes or due to a higher co-operative number (n). The latter possibility cannot be ruled out. However, the measured slopes of the log [Sr]–log release relations are between 1·0 and 3·0 for a range of concentration where Ca ions also produce similar slopes. Therefore, it was assumed that the same n applies to Sr and Ca. The minimal n that fitted the experimental data for Ca was 4 (Dodge & Rahamimoff, 1967), hence the same number was used for Sr.

After assuming that $n = 4$, it was possible to check whether the difference in potency is due to the affinity constant or the effectiveness. The results presented in Figs. 2, 3 and 5 show that the dissociation constants for Ca and Sr are similar, and that the main difference is in the effectiveness of the complexes: SrX is β times less effective than CaX. Fig. 10 shows the effect of various β on a theoretical relation between divalent ion concentration and the average number of quanta liberated per nerve impulse, assuming equal affinity constants. For example, when β is 0·4, the appropriate quantal content is only 2·56 % of the value at $\beta = 1{\cdot}0$. The suggested general equation describing the relation between the concentration of a divalent ion combining with X (MeX) and release is therefore

$$m = K\left(\frac{\beta_{Me}[Me]/K_{Me}}{1+[Me]/K_{Me}}\right)^n, \qquad (12)$$

where β_{Me} is the relative potency of the MeX complex compared to CaX, and K_{Me} is the dissociation coefficient of the $Me + X \rightleftarrows MeX$ reaction. As can be seen from Fig. 10, divalent ions with small β (less than 0·15) will produce negligible release. These ions will compete with Ca (or with Sr) on the postulated sites X, and serve therefore as competitive inhibitors. The effect of an inhibitor, present at a constant concentration throughout an experiment, is to change the apparent values of the dissociation constants. Therefore the [Na] term was omitted from the various equations (see Colomo & Rahamimoff, 1968). The equation relating the concentrations of the two activating divalent ions (Ca and Sr in this case) and release (eqn. 6), is based on the assumptions that Ca and Sr are acting on the same sites and that the two ions can co-operate. The fraction of sites occupied by Ca is given by: $CaX = ([Ca]/K_1)/(1+[Ca]/K_1+[Sr]/K_3)$ and by Sr: $SrX = ([Sr]/K_3)/(1+[Ca]/K_1+[Sr]/K_3)$. If the relative effectiveness is β, then the total fraction of active sites is $(MeX)_{active} = CaX + \beta SrX$. Quantal release is then: $m = K(MeX)^n_{active}$; the explicit form of this relation is given by eqn. (6).

The physical basis of the reduced effectiveness of SrX depends on the interpretation of what the critical releasing sites X are. There is strong

evidence that the entry of calcium ions through the presynaptic nerve membrane caused by depolarization is the important factor in the coupling between depolarization and transmitter release (Katz & Miledi, 1967). Recently the evidence was strengthened by experiments performed by Katz & Miledi (1969*b*) on the tetrodotoxin- and tetraethylammonium-treated stellate ganglion of the squid. In this preparation presumably an

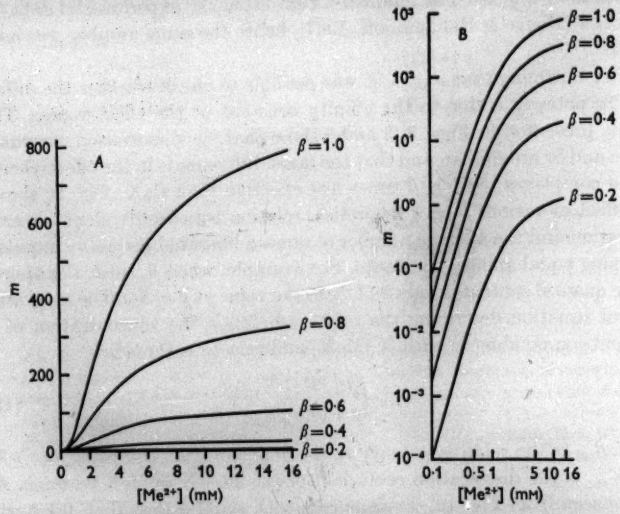

Fig. 10. Influence of β on the theoretical relation between m and activating divalent ion concentration. *A*, Linear co-ordinates; *B*, double logarithmic co-ordinates. The relations follow the equation

$$m = K\left(\frac{\beta_{Me}[Me]/K_{Me}}{1+[Me]/K_{Me}}\right)^4,$$

where $K = 1000$, $K_{Me} = 1{\cdot}0$ mM and β_{Me} has the successive values of 1·0, 0·8, 0·6, 0·4 and 0·2 (see text).

entry of Ca ions occurs, associated with transmitter release. This Ca hypothesis can be easily applied to our case. Assuming that the sites X are the channels through which Ca and Sr enter the nerve terminal, the similar affinity of both ions could be interpreted as equal probabilities of occupying the channel. If the important parameter is the concentration of the activating divalent ion on the inner side of the nerve membrane or the concentration *in* the nerve terminal, then it might be expected that the heavier Sr ions will move slowly, will create a lower concentration in the

relevant location and therefore will be less effective on a molar basis. It would be expected also that the reverse movement of Sr to the outer surface of the nerve membrane will be slower and therefore the residual increased probability of release after the e.p.p. will be prolonged (N. Moran & R. Rahamimoff, unpublished).

We are grateful to Sir Bernard Katz for valuable discussion and for his help in the preparation of the manuscript. We thank Miss E. Eilander for performing the radioactive experiments. This work was supported in part by a research grant from the Singer and Lunenfeld–Kunin funds.

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J. Physiol. (1971), **215**, *pp.* 727–741
With 6 text-figures
Printed in Great Britain

THE EFFECT OF TETANUS TOXIN IN THE GOLDFISH

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(*Received 4 November 1970*)

SUMMARY

1. The effects of tetanus toxin have been investigated both on central nervous and peripheral neuromuscular systems of goldfish.

2. Tetanus toxin kills goldfish when administered in minute doses. The lethal effect is temperature dependent. Unlike mammals, in which tetanus toxin produces spastic paralysis and convulsions, the tetanus-intoxicated goldfish die with an apparently flaccid paralysis.

3. Inhibition and excitation were investigated on the Mauthner cells of goldfish which had been paralysed with tetanus toxin for at least 24 hr, and which were subsequently kept alive for up to 3 days by perfusing the gills with oxygenated water. In such fish, antidromically and orthodromically excited Mauthner cells were quite normal, and there was no apparent effect on either the collateral inhibition or the crossed VIIIth nerve inhibition.

4. Local injection of sublethal doses of tetanus toxin into the pectoral fin muscles produced local paralysis of the fin. Nerve–muscle preparations were isolated from such goldfish; in tetanus toxin-paralysed fins, the muscle no longer responded to stimulation of its nerve. The nerve compound action potential was still present and the muscle still responded vigorously to direct electrical stimulation.

5. It is concluded that the major part of the lethal action of the toxin in the fish must be ascribed to a peripheral effect, the blocking of neuromuscular transmission. The inhibitory neuronal systems acting on the Mauthner cells of the goldfish, in apparent contrast to those acting on mammalian spinal neurones, are highly insensitive to tetanus toxin.

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INTRODUCTION

Botulinum and tetanus toxins, produced by the anaerobic sporing bacilli, *Clostridium botulinum* and *Cl. tetani* respectively, are both potent neurotoxins. It is generally considered that while botulinum toxin paralyses animals by blocking neuromuscular transmission, the primarily convulsive effects of tetanus toxin are due to a blocking of inhibition in the central nervous system (see Eccles, 1964). Botulinum toxin acts presynaptically at the neuromuscular junction, preventing the release of acetylcholine in response to nerve stimulation, and the quantal release which occurs spontaneously (Ambache, 1949; Brooks, 1956). The effect of tetanus toxin, however, is not nearly so well worked out. Brooks, Curtis & Eccles (1957) found that the toxin, like strychnine, increased the polysynaptic reflex responses in cats, leaving monosynaptic ones unaffected, and that it decreased five different types of inhibition which were investigated. The electrical responses on the surface of the spinal cord believed to result from activity in inhibitory interneurones were apparently unaffected, and it was suggested that the toxin probably exerted its effects at the inhibitory synapses on the motoneurones. Curtis & de Groat (1968) recorded Renshaw cell activity extracellularly, before and after the application of tetanus toxin from a micropipette situated close to the cell; the toxin prevented the inhibition (produced by squeezing the ipsilateral hind paw) of acetylcholine-induced and spontaneous Renshaw cell firing. In contrast, the inhibition of Renshaw cells by iontophoretically applied glycine (a putative inhibitory transmitter substance) was not blocked by tetanus toxin. Curtis & de Groat therefore suggested that tetanus toxin could be acting presynaptically at inhibitory synapses (but see Roper, Diamond & Yasargil, 1969).

The various mechanisms of synaptic transmission at the Mauthner cell of the goldfish have been analysed in considerable detail (Furshpan & Furukawa, 1962; Furukawa & Furshpan, 1963; Furukawa, 1966; Diamond, 1968). The cell can be relatively easily identified electrophysiologically, and micro-electrodes can be located with some accuracy in the morphologically distinct synaptic regions. The present study was initially undertaken to investigate the possible effects of tetanus toxin on physiological and pharmacological inhibition of Mauthner cells. However, although in preliminary experiments even minute doses of the toxin killed the fish, no abnormalities were detected in the various responses of these neurones even in acutely intoxicated fish. The fish died with an apparently flaccid paralysis associated with the cessation of respiration.

Now although the suppression of synaptic inhibition in the central nervous system is commonly accepted as responsible for the convulsions

and spasticity in tetanus-intoxicated animals (including birds (Davies, Morgan & Wright, 1955), reptiles (Cowles & Nelson, 1947) and frogs (Rowson, 1961)), there is evidence even in mammals for another, peripheral action of tetanus toxin, comparable to the neuromuscular blocking action of botulinum toxin (Kaeser & Saner, 1969; see also Ambache, Morgan & Wright, 1948*a*, *b*; van Heyningen & Mellanby, 1970). The apparently flaccid paralysis produced in goldfish, coupled with the apparent insensitivity of the central nervous system to the toxin, pointed strongly to a peripheral action of the toxin in fish.

The work reported here attempts to separate the possible central and peripheral actions of tetanus toxin. The results suggest that goldfish are particularly suitable for the study of the peripheral action of the toxin and may be relatively insensitive to its central effects. A preliminary report of these studies has been published (Diamond & Mellanby, 1970).

METHODS

Recording excitation and inhibition in the Mauthner cell

The methods are essentially those described by Furshpan & Furukawa (1962), Furukawa & Furshpan (1963) and Diamond (1968). The fish (*Carassius auratus* L.) contained in a special chamber, was perfused continuously through the mouth with water containing as an anaesthetic 0·25–0·3% urethane. The Mauthner axons were excited by a pair of steel wires, insulated except at the tip, which were positioned just outside the vertebral column. The VIIIth nerves were excited by a similar pair of electrodes inserted through holes in the skull. Mauthner cell activity was recorded with conventional glass KCl-filled micro-electrodes. The late collateral inhibition (L.C.I. of Furukawa & Furshpan, 1963) was revealed either as a hyperpolarization recorded in the Mauthner cell following the antidromic spike, and/or by the reduction in size of a second antidromic spike produced within 4–7 msec of the first. (This reduction is a useful measure of the post-synaptic inhibitory conductance change.) This inhibition characteristically disappears when the pair of stimuli are delivered at 5–10/sec, due to fatigue, and in this way its effect on spike height can be distinguished from any reduction of spike height due to refractoriness. The crossed VIIIth nerve inhibition was also measured either as a hyperpolarization of the Mauthner cell or by the reduction it caused in the amplitude of the antidromic spike.

The isolated nerve-muscle preparation

Fig. 1 shows the muscle which was used in the experiments *in situ* and the preparation as it was dissected out. It was mounted on a cork platform with a pin through the remaining piece of pectoral girdle, so that the *adductor superficialis* muscle hung vertically. A loop was secured through the base of the fin and attached to a fine chain suspended from the arm of a tension transducer. The output from the transducer was fed directly into a cathode ray oscilloscope. The nerve was stimulated with a pair of chlorided silver wire electrodes. Direct stimulation of the muscle was done by placing electrodes across it at the base and at the fin end, so that they made contact with as much of the surface as possible. The whole preparation was immersed in continuously oxygenated Ringer solution (NaCl 132 mM; KCl 3·1 mM;

$CaCl_2$ 3 mM; $MgCl_2$ 1 mM; Na-phosphate buffer 3 mM, pH 7; glucose 5·5 mM) except when being stimulated; such preparations could remain responsive for 24–48 hr after isolation from the fish. The muscles taken from fish weighing 60–80 g (length excluding tail, 14–20 cm) gave maximal twitch tensions of 5–20 g, and tetanic tensions of 10–40 g.

The compound action potential recorded from the electrically excited nerve to the fin muscle was displayed after amplification, on the screen of an oscilloscope which was photographed.

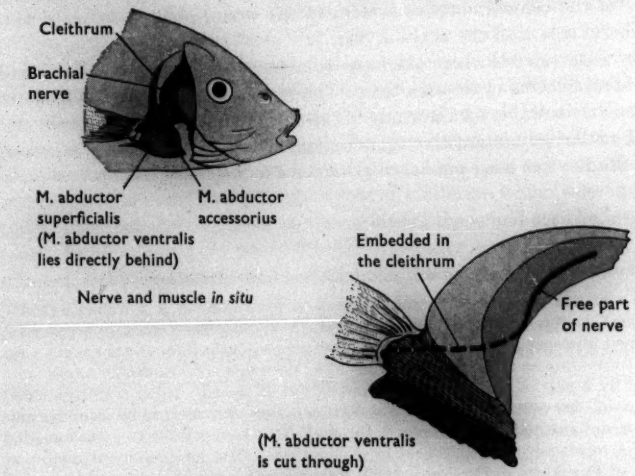

Fig. 1. The nerve–muscle preparation.

Tetanus toxin. The preparation, which was kindly provided by Dr R. O. Thomson of the Wellcome Research Laboratories, contained 60 million LD_{50}/mg and 360 L+ doses/mg. It was 41 % protein, the rest being made up of salts. The toxin was assayed by injecting serial twofold dilutions of the experimental solutions into pairs of Swiss albino mice. One LD_{50} was defined as the amount of toxin which would kill half the mice injected with it within 7 days.

Tetanus antitoxin solution (also obtained from Dr Thomson) contained 1800 Lf/ml.

Injections of fish. The preparations to be injected were dissolved in fish-Ringer solution containing 0·2 % gelatin as protective colloid. Injections were made at the base of the tail, into the belly of the pectoral fin muscles, or directly over the brain through a hole drilled in the skull.

RESULTS

Toxicity of tetanus toxin to goldfish

The toxicity of intramuscularly injected tetanus toxin, as measured either by the time to death following a given dose, or by the dose needed to

kill within a selected period, depends on the temperature of the goldfish. Fig. 2 shows the effects of temperature on the toxicity (as measured by time required to kill) of various doses of tetanus toxin. At 28° C, 20 mouse LD_{50} of tetanus toxin killed the fish within 5 days, whereas at 18° C this dose of toxin had no apparent effect on the fish within 50 days.

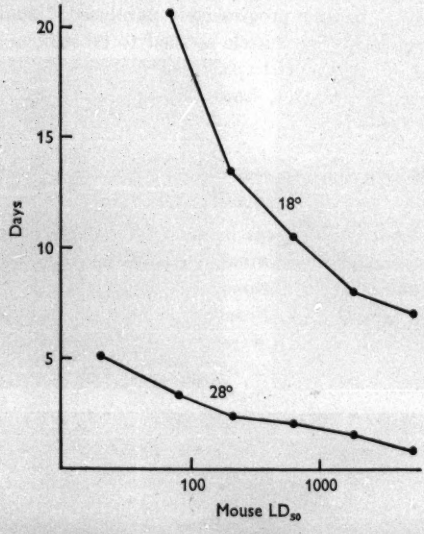

Fig. 2. Toxicity of tetanus toxin in goldfish. The abscissa shows (on a log scale) the dose of the tetanus toxin (measured in mouse lethal doses, LD_{50}) given intramuscularly; the ordinate shows the time taken for the fish to die. The two series of results were obtained from fish kept at the two indicated temperatures (18 and 28° C).

Symptoms of tetanus intoxication in goldfish

General. When fish had been injected (either at the base of the tail, or over the brain) with a 'lethal' dose of tetanus toxin, the earliest change in behaviour was occasional erratic swimming, particularly in response to a bang on the tank. During the following 2–3 days the fish became increasingly lethargic, and the respiratory movements of the operculi became spasmodic and less frequent; eventually the animals turned on to their sides and floated immobile. At this stage they sometimes responded to a bang on the side of the tank by normal, or sometimes erratic, swimming for a period of a few seconds. About 6–7 days after injection, breathing movements ceased

and they became completely paralysed. The heart often continued to beat for a few hours, but the animals always died within a day.

Local. If a single small dose (10–200 mouse LD_{50}) of tetanus toxin was injected into the pectoral fin muscles, an apparently localized effect of tetanus toxin was produced. After about 4 days (with 20 LD_{50}) at 18° C it was noticeable that the fin was held closer to the body than normal. Over the next few days it became progressively paralysed. Usually the fin was flaccid, but occasionally the muscle seemed to be stiff, and then the fin presented detectable resistance to passive movement, even when its motor nerves were cut. It was still, however, responsive to *direct* electrical stimulation (see below).

Investigations on the central nervous system of goldfish intoxicated with tetanus toxin

Initially the Mauthner cell was investigated in intoxicated fish whose swimming was markedly abnormal and erratic. Fig. 3 shows records obtained from one of the Mauthner cells of such a fish. In *A* the 'late collateral inhibition' or LCI (Furukawa & Furshpan, 1963) is demonstrated. Two antidromic spikes were fired, with an interval of a few msec between them; the second spike of the pair was markedly reduced in

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Legend to Fig. 3.

Inhibition of the Mauthner cell in intoxicated goldfish. A. The late collateral inhibition. Each record (upper and lower) shows two antidromically-excited spikes recorded intracellularly from one Mauthner cell. In the upper record a single pair of antidromic stimuli only was used. The lower record shows a number of superimposed traces like that above, but the pair of stimuli was repeated at 10/sec (the first few traces of the series were not photographed). The collateral inhibition is revealed as a reduction in amplitude of the second spike of the pair and is present in the upper record, but 'fatigued-out' in the lower (see text for further description).

B. The crossed VIIIth nerve inhibition. Upper record, single antidromically-excited Mauthner cell spike. Lower record, the same spike but now preceded by a stimulus to the opposite VIIIth nerve (the now reduced spike is superimposed on a slow positivity; see Furukawa & Furshpan, 1963).

C. VIIIth nerve excitation and inhibition. On the left, the Mauthner cell spike fires from an e.p.s.p. which is indicated by the arrow, and which follows the large stimulus artifact. On the right, the same orthodromic excitation is preceded by a stimulus to the opposite VIIIth nerve (compare *B* above). The spike is absent, and a subthreshold e.p.s.p. is left (marked by the arrow).

Calibrations: vertical: 5 mV (*A*, *B* and *C*); horizontal: 1 msec (*A* and *C*); 0·5 msec (*B*).

amplitude (Fig. 3*A*, upper record). This reduction is due to the activation of the collateral inhibition, and is the consequence of the reduced input resistance of the Mauthner cell (i.e. the reduction in spike amplitude reveals the inhibitory conductance increase of the post-synaptic membrane). In the lower record the pair of stimuli were repeated 10 times a second, at which frequency the inhibition fatigues; the two antidromic

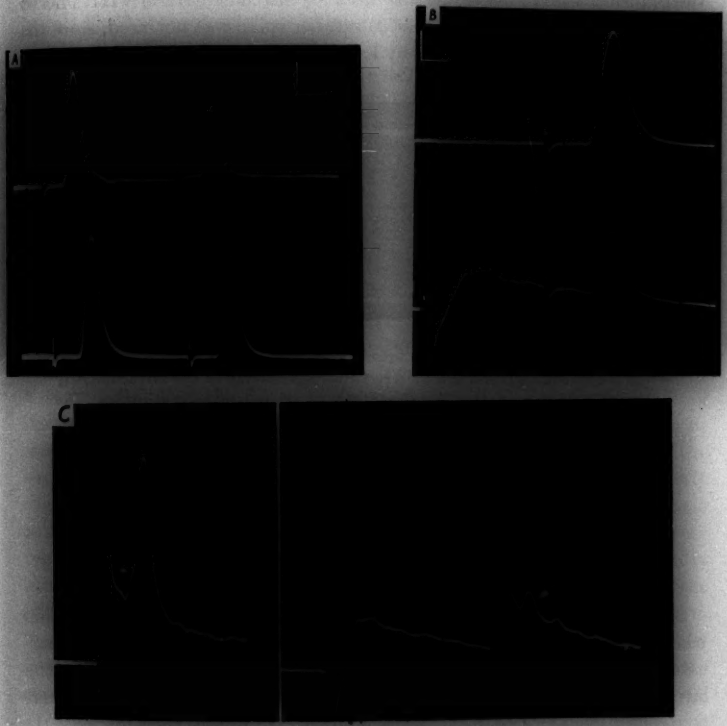

Fig. 3. For legend see opposite page.

spikes were now similar in size. (The small reduction in the second spike was due to refractoriness of the spike-generating mechanism.) Fig. 3*B* demonstrates a similar reduction in the height of an antidromic spike, in this instance when it occurred a few milliseconds after stimulation of the opposite VIIIth nerve; the VIIIth nerve was shown by Furukawa & Furshpan (1963) to exert predominantly an inhibitory influence on the

contralateral Mauthner cell. Fig. 3*C* shows this crossed VIIIth nerve inhibition acting on an *orthodromically*-excited Mauthner cell, i.e. fired by a stimulus applied to the *ipsilateral* VIIIth nerve. The first record on the left shows the orthodromic spike alone; in contrast to the antidromic spike it is initiated by a clearly defined excitatory post-synaptic potential (e.p.s.p.). The second record shows the inhibition caused by excitation of the opposite VIIIth nerve. In this instance the contralateral VIIIth nerve inhibition was very powerful: the Mauthner spike was not fired, since the much-reduced e.p.s.p. (marked by an arrow) was well below threshold.

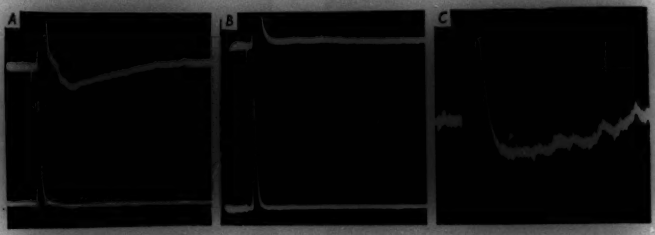

Fig. 4. Inhibitory potentials in intoxicated fish. *A* and *B*. The upper and lower traces are high and low gain records respectively of the intracellularly recorded response of the Mauthner cell to an antidromic stimulus. The inhibitory hyperpolarization in *A* (following the Mauthner cell spike) is absent in *B*, which shows superimposed traces of antidromic responses repeated at 10/sec (see text).

C. The arrow indicates the hyperpolarization produced in the Mauthner cell by excitation of the *opposite* VIIIth nerve. The initial upward deflexion is the stimulus artifact.

Calibrations: vertical: 1 mV and 10 mV (*A* and *B*) and 0·2 mV (*C*); horizontal: 5 msec (*A* and *B*) and 2 msec (*C*).

Similar records to those shown in Fig. 3 were obtained on all occasions when the Mauthner cell was investigated in behaviourally abnormal, intoxicated, fish. These results, both of inhibition (the collateral *and* the contralateral VIIIth nerve) and of excitation (orthodromic and antidromic) were completely characteristic of normal goldfish. It appears therefore that the apparent 'convulsions' were occurring without any detectable effect on at least these particular inhibitory (and excitatory) systems acting on the Mauthner cell.

All fish which had been injected with a lethal dose of tetanus toxin eventually died with a flaccid paralysis, and many of these died without an intervening period of 'convulsive' behaviour as described above. Mauthner cells were also investigated in a number of fish which were dying in the flaccid condition. Again, in these fish, the inhibition and

excitation of the Mauthner cell was completely normal. Sometimes the inhibitory conductance increase of the Mauthner cell is associated with a hyperpolarization of up to about 2 mV (Furukawa & Furshpan, 1963). Fig. 4 shows records obtained from a Mauthner cell in a flaccidly paralysed fish, and demonstrates that this hyperpolarizing inhibitory post-synaptic potential could still be present. In records *A* and *B*, an antidromically excited spike is shown at low gain (lower trace) and at high gain (upper trace). The hyperpolarization which occurred during the period when the collateral inhibition was shown to act can be clearly seen following the spike in the upper record of Fig. 4*A*. In 4*B* the antidromic stimulation was repeated at 10 times a second, when the collateral inhibition fatigues out; the hyperpolarization (and the inhibition) was now absent. Fig. 4*C* shows a hyperpolarization (indicated by the arrow) following the excitation of the contralateral VIIIth nerve, which caused the type of inhibition shown in Fig. 3*B* (this inhibitory potential is preceded by a large stimulus artifact). Unlike the collateral inhibition, the crossed VIIIth nerve inhibition does not fatigue appreciably even at stimulation frequencies of 10/sec or more.

Direct application of toxin to the brain

Since the above experiments had revealed no abnormalities in the various excitatory and inhibitory systems acting on the Mauthner cell, it was possible that the fish were dying of a peripheral paralytic action of the toxin before any central action had had time to occur. In order to be sure that a lack of central effects was not due merely to an inability of the toxin to enter the central nervous system, the toxin was injected directly over the brain through a hole drilled in the skull. In order to give the toxin as much time as possible to act on the central nervous system, the fish were kept 'alive' after they had become totally paralysed, by continuously perfusing them with aerated water into the mouth and out through the gills. Under these circumstances, in some fish the heart would continue to beat for several days (although in many cases it would stop after a few hours). In this way Mauthner cells were investigated in fish which had been completely paralysed for up to 3 days.

However, even in these fish no abnormalities in either inhibition or excitation of the Mauthner cells were detected; collateral inhibition and contralateral VIIIth nerve inhibition were found to be acting entirely normally. Fig. 5 illustrates that the principal mechanism underlying the inhibition was also still working normally. In this experiment large ($1\frac{1}{2}\,\mu$ tip diameter) KCl-filled micro-electrodes were used, so that there was a marked diffusion of chloride into the cell. The mechanism of chemically transmitted, post-synaptic inhibition of the Mauthner cell, as in so many neurones, involves a striking increase in the chloride conductance of the

membrane, and in these experimental circumstances the activation of the inhibitory synapses results in depolarization of the cell (Furukawa & Furshpan, 1963). If this depolarization is large enough, it can act as an e.p.s.p., and itself initiate a spike, i.e. the inhibition can be converted into an excitation; this phenomenon is entirely dependent on the attainment of a large enough concentration of chloride inside the cell, and then will occur in normal Mauthner cells, as in normal motoneurones of the cat

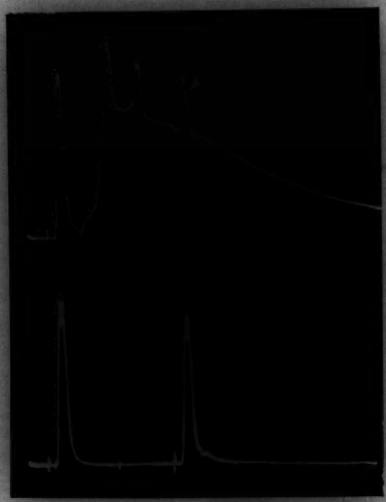

Fig. 5. The collateral 'inhibition' during Cl diffusion into the Mauthner cell in intoxicated fish. The effect of the increased intracellular concentration of Cl is to convert the inhibition into an excitation. The cell was excited antidromically by a pair of stimuli each of which fired a spike. The second spike is indicated by the arrow in the upper record. The two intervening spikes in the upper record were caused by the enormous depolarization attributable to activation of the collateral inhibitory systems. The lower record consists of superimposed traces of the response when the pair of stimuli was delivered at 10/sec (cf. Fig. 3 and text).

Calibrations: vertical: 5 mV; horizontal: 2 msec.

spinal cord (Coombs, Eccles & Fatt, 1955). In the upper record of Fig. 5 the collateral inhibition which follows the antidromic spike was converted into an excitatory depolarization in this way, and in this instance itself fired two spikes. The spike marked by an arrow was produced by a second antidromic stimulus. In the lower record the pair of antidromic stimuli was repeated at 10 times a second, at which frequency the collateral inhibition fatigued out, the chloride conductance change was no longer

produced, and hence the depolarization was absent. The two antidromic spikes now had their usual characteristics (cf. Fig. 3*A*).

Peripheral effects of tetanus toxin

Fish were injected with 200 LD_{50} (mouse) of tetanus toxin into the pectoral fin muscle on one side, and the same amount of toxin, mixed with a neutralizing dose of antitoxin, was injected into the corresponding muscle of the other side. This dose of toxin, at 14° C, is sublethal. The fish

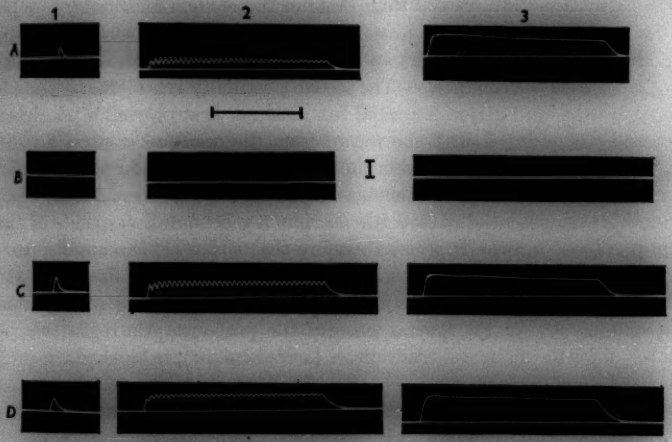

Fig. 6. Muscle tension records from tetanus toxin-injected fish. Records from right (*B* and *D*) and left (*A* and *C*) fins of same fish. The right fin muscle was injected with tetanus toxin and the left with tetanus toxin mixed with a neutralizing dose of antitoxin. *A* and *B* show responses to stimulus of the nerves; *C* and *D* show responses to direct stimulation of the muscle. Left-hand columns, single maximum twitch. Middle columns, tetanic stimulation at 16/sec. Right-hand columns, tetanic stimulation at 60/sec.

Calibrations: vertical: 20 g; horizontal: 1 sec.

were killed at various times after the injection when some abnormality was visible in the movement of the fin, and the fin muscle and its nerve then dissected out from each side. The preparation was set up so that the nerve or the muscle could be stimulated electrically, and the mechanical response of the muscle recorded.

At about 4 days after the local injection of toxin the mechanical response to nerve stimulation was always weaker on the toxin-injected side; by 6 days the muscle barely responded, or responded not at all, to stimulation of its nerve (Fig. 6*A* and *B*). Paralysed fin muscles, left *in vivo*, did not

recover during periods of observation of which the longest was 30 days. The response of the muscle to direct electrical stimulation was tested immediately after recording the response to stimulation of the nerve. All the muscle still responded to direct electrical stimulation (Fig. 6*C* and *D*). Thus the weakened or absent response to stimulation of the nerve in intoxicated muscles was not due to a failure of the muscular response itself. However, in preparations which had been paralysed for 3 or more days before their isolation from the animal, the responses to direct electrical stimulation were always less than those of the control muscles.

Since the toxin-paralysed muscles responded to direct stimulation, the paralysis could be due either to a block of neuromuscular transmission or to a block in nerve conduction. The second possibility was tested by recording the compound action potential from the nerve supplying a paralysed fin, and comparing it with the record from the opposite, control fin. No marked differences were found in the size or shape of the compound action potentials. The stimulus parameters used to obtain this *maximum* compound action potential caused the *maximum* mechanical response in the non-paralysed muscle. It seems therefore that either (1) the impulse conduction in the pre-terminal portions of the motor nerve is affected by the tetanus toxin, and/or (2) there is prevention of acetylcholine release from the endings and/or (3) the response of the post-synaptic region of the muscle fibre to the neural transmitter (acetylcholine), is blocked.

DISCUSSION

The inhibitory systems acting on the Mauthner cells of goldfish appear to be insensitive to the action of tetanus toxin, which produces such dramatic effects in mammals. However, the toxicity of tetanus toxin to goldfish is comparable to its toxicity to mice. Thus at 28° C a 60 g fish will die within 5 days from only 20 times the weight of toxin which would take the same time to kill a 20 g mouse (at 38° C). This contrasts markedly with the frog, which (at 28° C) is at least 1000 times less sensitive to tetanus toxin than is the mouse.

The goldfish apparently dies from a peripheral, paralytic action of tetanus toxin, which seems superficially comparable with the action of botulinum toxin. It has been found in preliminary experiments (J. H. Mellanby, unpublished observations) that botulinum toxin injected into the fin of goldfish produces symptoms indistinguishable from those produced by tetanus toxin. Twenty years ago the two toxins were shown to have similar effects on another cholinergic system, in the rabbit. Thus Ambache *et al.* (1948*a*) (see also Mellanby, Pope & Ambache, 1968) showed that both toxins, when injected into the anterior chamber of the eye, pre-

vented the response of the *sphincter pupillae* muscle to stimulation of the oculomotor nerve (which is preganglionic).

There is other evidence in the literature suggesting that under some circumstances it is possible to produce a flaccid instead of a spastic paralysis with tetanus toxin. Thus Davies & Wright (1955) found that when mice were injected intravenously with a large dose of tetanus toxoid (330 Lf) at the same time as they were given tetanus toxin (36 LD_{50}) they could be protected from the tetanus toxin. If the dose of toxoid was insufficient to protect the mice completely, then there were no convulsions or muscular spasms, and instead the muscles appeared to be flaccid. Miyasaki *et al.* (1967) reported that if an intramuscular injection of tetanus toxin in rabbits is followed 2 hr later by intravenous injection of antitoxin, a predominantly flaccid paralysis develops. Similar flaccidity was also produced when the toxin was injected into the epineurium of the ischiadicus nerve. These authors concluded that tetanus toxin has two pharmacological actions, one central and one peripheral. A similar conclusion was reached by Prabhu, Oester & Karczmar (1962). Prabhu & Oester (1962) suggested that in rabbits, intramuscularly injected tetanus toxin has a dual action – a central one occurring first, followed after some days by a local neuromuscular blocking action on the injected muscle. Muchnik & Rubinstein (1967) found that with the rat isolated phrenic–hemidiaphragm preparation tetanus toxin blocked the mechanical response of the muscle to stimulation of its nerve $\frac{1}{2}$–3 hr after it was added to the bath. Surprisingly, since they did not record the response of the muscle to direct stimulation, they concluded that the toxin interfered directly with the contractile mechanism in the muscle.

Our conclusion from the present experiments on goldfish is that tetanus toxin has a peripheral site of action, probably at the neuromuscular junction, and that goldfish die as a result of the paralysis caused by this action. We have no direct evidence that in the goldfish the toxin has any central action at all; the occasional erratic swimming seen in intoxicated fish could be attributable to the disorganization of movement, and therefore of sensory feed-back, caused by the peripheral action of the toxin. Although we have some preliminary observations suggesting a possible direct action of the toxin on the muscle itself it seems that the major part of the effects can be attributed to blocking of transmission between nerve and muscle. In preliminary experiments the miniature end-plate potentials, which are readily recorded in normal fin muscles, appear to be absent in paralysed ones (J. H. Mellanby and P. Thompson, unpublished). Further experiments are in progress to check whether the effect of the toxin is largely presynaptic, as in the case of botulinum toxin, or whether there is an associated reduction in post-synaptic sensitivity to acetylcholine. The

absence of any central action of the toxin is surprising but we have not excluded the possibility that the peripheral system is so highly sensitive to tetanus toxin in the goldfish that there is not enough time for a central action to be manifested. Nevertheless, even when the toxin was applied directly over the brain and the animal was kept alive for as long as 72 hr after the total peripheral paralysis had occurred, we were still unable to detect any interference with excitation or inhibition acting on the Mauthner cells.

The apparent differences between the susceptibility to tetanus toxin of inhibition acting on the Mauthner cell and of that acting on spinal neurones in the cat are surprising. Both inhibitions are blocked by strychnine; both are mimicked by glycine, whose action is also prevented by strychnine. In a recent report it was suggested that strychnine could be acting presynaptically on the systems inhibiting the Mauthner cells, or possibly that glycine may not be eligible as the inhibitory transmitter at the Mauthner cell (Roper *et al.* 1969; Roper & Diamond, 1970). The evidence for the motoneurone has been interpreted very strongly in favour of glycine as the inhibitory transmitter (Curtis & Johnston, 1970). Our feeling is that at present we cannot distinguish between the possibilities (1) that there are subtle but important differences between the fish and the mammalian inhibitory systems, and (2) that the apparent differences are dependent on the differences of the experimental conditions in which similar techniques were used. Certainly, in the present experiments the inhibition of the Mauthner cells in tetanus-intoxicated fish was readily abolished by strychnine. Until comparative data are available for different neurones in different parts of the brain of different animal types, it may be important not to generalize from results obtained from relatively few kinds of neuronal inhibitory systems.

We wish to acknowledge with thanks the technical assistance of Mr P. Thompson, and the support kindly provided by the Medical Research Council and the Muscular Dystrophy Group of Great Britain.

We would also like to thank Mr E. G. Huckin for drawing the Figures.

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J. Physiol. (1971), **215**, *pp.* 743–753
With 4 text-figures
Printed in Great Britain

THE DISCHARGE OF SWEAT IN WELSH MOUNTAIN SHEEP

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(*Received 24 November 1970*)

SUMMARY

1. Experiments were done on Welsh Mountain sheep to determine whether the sheep can be caused to discharge sweat continuously.

2. On administration of sequences of four to eighteen I.V. injections of adrenaline or noradrenaline (200 μg), sheep responded to the first 4–5 injections with decreasing amounts of sweat discharge. Thereafter, the responses were fairly constant at about 10% of the first discharge. Occasionally one injection within a series elicited no sweat discharge but on other isolated occasions large discharges of sweat were induced. The blood pressure response to repeated I.V. injections of adrenaline remained constant.

3. Inhalation of amyl nitrite or rebreathing of expired air during I.V. adrenaline injections had little effect on the pattern of intermittent sweat discharges and did not induce a continuous outflow of fluid from the sweat glands.

4. Continuous infusions of adrenaline and of carbachol did not cause a change in the rate of continuous water vapour loss from the skin but possibly increased the frequency of intermittent sweat discharges.

5. Electrical stimulation of the cervical sympathetic nerve for short (0·5–2 min) or long (20–30 min) periods caused only transient (3–6 min) increases in water vapour loss from the cheek skin.

6. None of these experiments indicated that the sweat glands of the sheep can be induced to discharge fluid continuously.

INTRODUCTION

The intermittent discharge of sweat in sheep which occurs over the trunk skin (Bligh, 1961) and on the scrotum (Waites & Voglmayr, 1963) is controlled by an α-adrenergic mechanism (Robertshaw, 1968). A discharge of sweat apparently identical with the natural discharge can be induced by I.V. adrenaline, 1–5 μg/kg body wt., or by slightly larger doses of noradrenaline (Bligh, 1961; Waites & Voglmayr, 1963; Allen & Bligh, 1969).

Allen & Bligh (1969) found that the rate of cutaneous water loss between the intermittent pulsatile discharges of sweat in sheep was no greater than could be attributed to simple diffusion of water through the skin. Hofmeyr, Guidry & Waltz (1969) also showed that after each sweat discharge in sheep the rate of evaporation of water from the skin returned to a basal level. However, several reports have indicated that an elevation of the rate of water vapour loss from sheep skin may occur under some circumstances. Immediately after shearing, Hofmeyr *et al.* (1969) recorded a considerable increase in surface evaporation which was not associated with pulsatile discharges of sweat. Hayashi (1968) reported that continuous intravenous adrenaline infusion in sheep elicited a 'strong sweat response' which began soon after the start of infusion and rapidly extended over all the areas tested for sweating. An elevated rate of water vapour loss on which intermittent sweat discharges were superimposed was recorded by Waites & Voglmayr (1963) from the scrotal skin of a ram after bilateral lumbar sympathectomy.

Bligh (1967) suggested that the pattern of sweating in sheep may be due to a combination of two mechanisms; (i) a slow secretion of sweat into the lumen of the glands, and (ii) a periodic expulsion of the lumen contents on to the skin surface due to a contraction of the myoepithelium around the glands. The present experiments were intended to test the validity of this hypothesis, and to determine whether there are any circumstances in which the rate of secretion is sufficient to cause a continuous outflow of sweat in sheep such as observed in certain other species by Allen & Bligh (1969). Preliminary results of some of the experiments have been reported briefly (Johnson, 1970).

METHODS

The experiments were done on unshorn mature Welsh Mountain wethers weighing between 27·5 and 37·0 kg. Drugs were administered intravenously via a polyethylene cannula implanted aseptically in the jugular vein at least 1 day before an experiment. For recording blood pressure a polyethylene or polyvinylchloride cannula was implanted in the common carotid artery by aseptic surgery under general anaesthesia at least 3 days before an experiment. Arterial and venous cannulae were flushed with heparinized saline every second day.

In the first three experimental procedures, the arterial cannula was connected to a pressure transducer (Consolidated Electrodynamics) and recorder (Devices) for measurements of blood pressure. Skin temperatures were measured at two points on the trunk beneath the fleece and at one point on each ear with polyethylene-covered copper–constantan thermocouples held on to the skin under pieces of rubber dam fixed with Latex adhesive. Skin temperatures and the temperature measured with a thermocouple probe at a depth of 10 cm within the rectum were registered on a multi-channel potentiometric recorder (Cambridge Instruments, 1 mV full-scale deflexion) once every 72 sec. Evaporative water loss was measured from one midside of the trunk with the ventilated capsule system described by Allen & Bligh (1969) and

from the other mid side of the trunk with a sudorimeter (Custance, 1962). The Custance sudorimeter regulates the air flow through a capsule on the skin so as to maintain the relative humidity beneath the capsule at 45 %. The rate of air flow which is recorded continuously is therefore proportional to, and determined by, the cutaneous water loss beneath the capsule. In the fourth experimental procedure, water vapour loss from each cheek was measured with a ventilated capsule and the skin temperature of each ear was recorded with a thermocouple.

Each sweat-measuring system was calibrated by injecting water beneath the capsule while it was attached to a thermally regulated plate. For both systems, the area traced out by the recorder after injection of a known amount of water was linearly related to the volume of water injected (Fig. 1) and estimates of the magnitude of sweat gland discharges in experimental records were derived from regression equations for these relations.

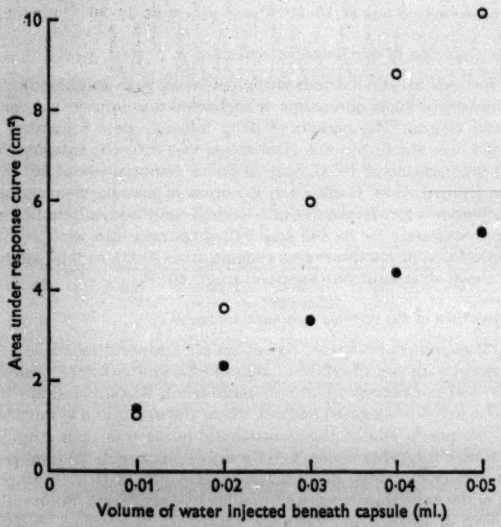

Fig. 1. Calibration of Custance and ventilated capsule sweat-measuring systems at an ambient temperature of 40° C and a plate surface temperature (equivalent to skin temperature) of 39° C. ●, Custance system; ○, ventilated capsule system.

Repeated I.V. *injections of adrenaline or noradrenaline*

Sequences of between nine and eighteen injections of adrenaline (each 200 μg base) were given I.V. to two sheep (27·5 and 28·5 kg body weight) in each of thirteen separate experiments. In seven of these experiments, adrenaline injections were given at intervals of 4–9 min throughout the experimental period. In the other six experiments, sequences of three to four injections were given at intervals of 4–9 min after which there was an interruption of 20, 40 or 60 min followed by a further three to four

injections, another interruption, then further injections, and so on up to a total of fifteen injections. In two other experiments, one on each sheep, series of four and twelve injections of noradrenaline (each 200 μg base) were given I.V. as in the first seven experiments with adrenaline. The experiments were done at an air temperature of 30 ± 2 °C.

Amyl nitrate inhalation and rebreathing

In eight experiments on three sheep (six done in conjunction with experiments described above), the effects of inhaling amyl nitrite and of rebreathing expired air were investigated. In inhalation experiments, amyl nitrite (0·2–0·6 ml.) was expelled from a syringe into cotton-wool placed loosely across the inlet valve of a face mask for 4–8 min. In rebreathing experiments, a flow of air normally pumped past the single outlet of a face mask was turned off for 5–10 min while the animal breathed through two corrugated rubber tubes each 1 m long and 2·2 cm i.d. Some of the experiments were carried out at 15–20° C and others at 29–30° C air temperature.

Continuous I.V. *infusion of adrenaline or carbachol*

In twenty-one experiments on four sheep, sweating rate and blood pressure were continuously measured while adrenaline or carbachol was infused I.V. for periods of between 15 and 30 min. The periods of drug infusion were separated by similar periods during which sterile physiological saline was infused. Infusions were made once at an air temperature of 10° C, once at an air temperature of 40° C, and again at an air temperature of 40° C after I.V. injection of phenoxybenzamine (3 mg/kg body wt.), 30–60 min before heat exposure. In each condition, adrenaline was infused at rates of approximately 0·125, 0·5 and 2·0 μg/kg.min into each of three sheep. Carbachol was infused at six dose rates ranging from 0·021 to 0·67 μg/kg.min into each of three sheep at an ambient temperature of 40° C.

Electrical stimulation of the cervical sympathetic nerve

In four acute experiments carried out at an air temperature of 30 ± 2° C, sheep were anaesthetized with pentobarbitone sodium (one animal) or chloralose-urethane (three animals) and the vago-sympathetic nerve trunk was exposed low in the neck. The sympathetic nerve was ligated and cut where the vagus and sympathetic nerves separate. The peripheral end of the sympathetic nerve was then stimulated using platinum electrodes for either short (0·5–2 min) or longer (20–30 min) periods with square pulses usually of 1 msec duration and a strength of 10 V at 10 per sec.

Drugs used in the experiments were adrenaline hydrochloride (Solution of Adrenaline 1/1000, May & Baker), noradenaline bitartrate (Levophed 1/1000, Bayer), carbachol (B.D.H.) and phenoxybenzamine (Dibenyline, S.K.F.). I.V. injections were washed into the vein with 0·5 ml. sterile physiological saline.

RESULTS

Effects of repeated I.V. *injections of adrenaline or noradrenaline*

The first four or five injections of adrenaline given in each experiment usually elicited progressively diminishing sweat discharges. Subsequent injections of adrenaline elicited sweat discharges of variable size, averaging about one-tenth of that elicited by the first injection of adrenaline. Sweat discharges were still recorded after as many as eleven, thirteen and eighteen

adrenaline injections, although sometimes, towards the end of a long series of injections, adrenaline failed to elicit a measurable sweat response. Occasionally one of the injections of adrenaline within a series would induce a sweat discharge of much greater than average size. For example in the experiment shown in Fig. 2, the last of a series of eighteen adrenaline injections given over a period of 125 min elicited a sweat discharge which was only just measurable, whereas the sixteenth injection induced a sweat discharge which was as large as that produced by the first adrenaline injection. Noradrenaline was as effective as adrenaline in inducing sweating responses, and the pattern of discharges following repeated injections of noradrenaline was similar to that recorded with adrenaline.

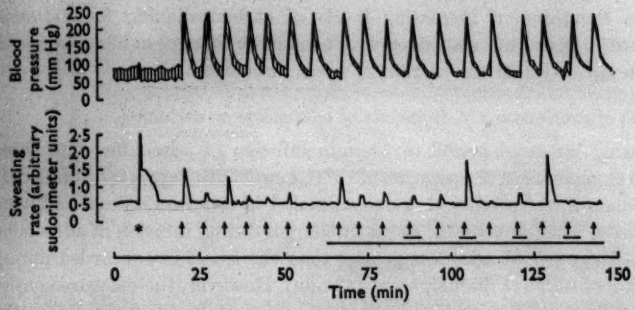

Fig. 2. Sweating and blood pressure responses to repeated injections of adrenaline in one sheep at an ambient temperature of 30° C. 200 μg adrenaline injected I.V. at each arrow. *, spontaneous sweat discharge. Single underline, face mask on sheep; double underline, amyl nitrite inhalation.

When series of adrenaline injections were interrupted for periods of 20, 40 or 60 min, the first injection after an interruption generally elicited a larger sweat discharge than did the last previous injection. With the second injection after the interruption, the volume of sweat discharged returned approximately to the amount recorded before the interruption. There were no consistent differences between the amounts of sweat discharged after delays of 20, 40 or 60 min.

Intravenous injections of adrenaline increased the systolic blood pressure from 90–130 mm Hg to 200–250 mm Hg or more (Fig. 2). In response to some of the early administrations of adrenaline the systolic blood pressure exceeded the measuring range of the recorder (250 mm Hg); however judging by the concomitant changes in diastolic pressure and the subsequent fall in systolic pressure (Fig. 2), the rise above 250 mm Hg was only slight. Repeated administration of constant amounts of adrenaline

elicited equal or only slightly diminishing rises in blood pressure, and following each intravenous injection of 200 μg adrenaline, systolic blood pressure increased in all cases to at least 200 mm Hg.

Effects of amyl nitrite inhalation and rebreathing

Amyl nitrite inhalation was followed in some experiments by an increase in ear temperature and a slight fall in blood pressure but in other experiments by no change; heart rate showed no consistent change. Amyl nitrite inhalation for 3–5 min before an intravenous injection of adrenaline did not consistently increase the volume of sweat discharged after the adrenaline injection, although such an effect was observed on isolated occasions (Fig. 2).

The breathing of increasing levels of carbon dioxide for 5–10 min (rebreathing) was not associated with consistent changes in blood pressure, skin temperature or the pattern of sweat discharges.

Effects of continuous I.V. *infusions of adrenaline or carbachol*

During the total period of 244 min infusion of adrenaline into three sheep at an ambient temperature of 10° C, 4 sweat discharges were observed; 3 discharges were observed in one animal, 1 in a second animal and none in a third. The frequency of sweat discharges during infusion of adrenaline (1 discharge per 61 min) was greater than the frequency recorded during infusion of saline (1 discharge per 148 min). However, the measurement of frequency is subject to large sampling errors at low frequencies so the significance of the difference is uncertain. Between the discrete discharges of sweat the background rate of cutaneous water loss during the infusion of either adrenaline or saline was in the range 8–32 g/m^2.hr.

At an ambient temperature of 40° C the frequency of discharge of sweat was considerably greater than at 10° C. At 40° C, 8 discharges of sweat were observed during the total period of infusion of adrenaline in the three sheep (1 discharge per 32 min) and 4 discharges were observed during infusion of saline (1 discharge per 50 min). The rate of cutaneous water loss between the sweat discharges returned to the background level measured at an air temperature of 10° C and was unaffected by the infusion of adrenaline even at rates sufficient to cause a marked elevation of blood pressure (Fig. 3).

Administration of phenoxybenzamine before heat exposure blocked the intermittent discharge of sweat (incompletely in one sheep), but there was no increase in cutaneous water loss above the background rate observed during infusion of either adrenaline or saline.

During the infusion of carbachol and the associated infusion of saline at an air temperature of 40° C, a similar pattern of sweat discharge was

observed to that seen during infusion of adrenaline and of saline at an air temperature of 40° C. The background rate of cutaneous water loss between the intermittent discharges of sweat did not increase at any time even though, during the higher rates of infusion of carbachol, systolic blood pressure and pulse pressure increased and the animals became restless and salivated profusely. The frequencies of discharge of sweat were considerably higher both during the infusions of carbachol (1 discharge per 19 min) and during the infusions of saline alternating with carbachol (1 discharge per

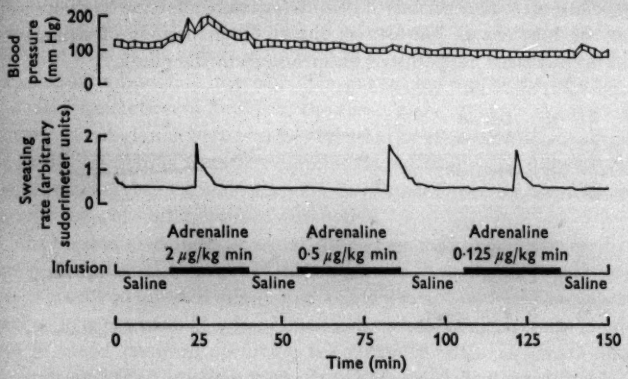

Fig. 3. Effect of I.V. infusions of saline and adrenaline on sweating rate and blood pressure in one sheep at an ambient temperature of 40° C.

21 min) than during earlier infusions of adrenaline or saline into the same animals. The small difference between the frequencies of sweat discharge during infusions of carbachol and of saline does not necessarily indicate that carbachol was without effect on the frequency of sweat discharge. The frequency of sweat discharge appears to have increased not only during the infusions of carbachol but also during the alternating infusions of saline, possibly due to the slow rate of inactivation of carbachol after systemic administration.

Effect of electrical stimulation of the cervical sympathetic nerve

When the peripheral end of the left cervical sympathetic nerve trunk was stimulated for short periods (0·5–2 min), the left eye protruded from its socket and the temperature of the left ear decreased towards air temperature. At the same time there was a small but distinct increase in evaporative water loss from the left cheek (Fig. 4). Evaporative water loss from the cheek returned to the level measured prior to stimulation within 3–6 min. No changes were observed on the unstimulated side of the animal. When

the stimulus was applied continuously for 20–30 min, ear temperature remained depressed and the eye on the stimulated side protruded throughout the entire period of stimulation. Evaporative water loss from the cheek on the stimulated side increased but the increase was only temporary and persisted for approximately the same time as that seen during periods of stimulation lasting only 0·5–2 min (Fig. 4).

Repeating the periods of stimulation of the cervical sympathetic nerve generally led to a slight diminution of the resultant sweat discharge. Nevertheless, up to four periods of stimulation were effective on each sheep, and on the four sheep, fourteen of the eighteen periods of stimulation elicited an increased evaporative water loss from the cheek.

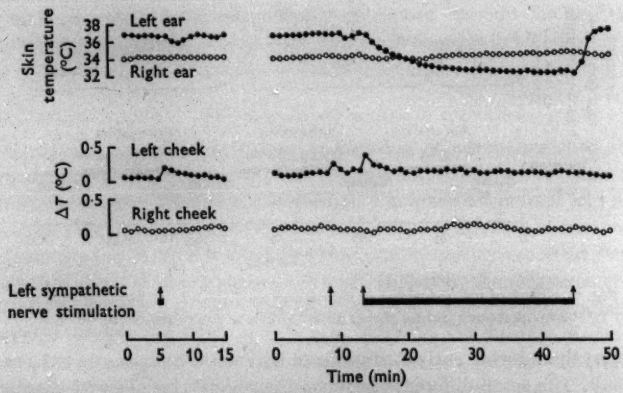

Fig. 4. Evaporative water loss from the cheeks (ΔT), and ear skin temperatures of one sheep during stimulation of the left sympathetic nerve (10/sec, 10 V, 1 msec) at an ambient temperature of 28° C.

DISCUSSION

Allen & Bligh (1969) have shown that in the ox, horse, donkey and llama exposure to heat and I.V. injections or infusions of adrenaline cause a sustained outflow of fluid from the sweat glands. It has not been possible in the present experiments to find any stimuli which lead to a similar continuous outflow of sweat in sheep.

Intravenous injections of adrenaline given repeatedly at short intervals each produced a discharge of sweat except on a very few occasions. Each discharge lasted for only a short time, after which cutaneous water loss returned to the background level measured prior to adrenaline administration. The first four or five injections of adrenaline elicited progressively

smaller discharges of sweat whereas subsequent injections elicited small and variable sweat discharges (Fig. 2). A similar pattern of sweat discharge was seen in sheep exposed to heat and has been described previously by Robertshaw (1968). An explanation for this pattern could be that sweat stored in the sweat gland lumen is depleted during the first few discharges and that sweat newly secreted into the lumen is expelled by later injections of adrenaline or later heat exposure. However, in the present experiments large discharges of sweat were sometimes produced by injections of adrenaline which had previously produced only small discharges (Fig. 2). Furthermore, long delays in a sequence of adrenaline injections were no more effective than short delays in increasing the amount of sweat subsequently expelled by adrenaline injection. The conclusion can be drawn that either the rate of secretion of fluid into the sweat gland lumen was very variable and the sweat glands were possibly emptied by each injection of adrenaline, or the sweat glands were not emptied by adrenaline injections and were simply discharging variable proportions of their lumen contents in response to injections of equal doses of adrenaline.

The decline in volume of sweat discharges induced by repeated I.V. injections of adrenaline could be the result of a decrease in sensitivity of the sweat glands to injected adrenaline, but it was not associated with a similar decline in sensitivity to adrenaline in the cardiovascular system since the rise in blood pressure occurring concurrently with the sweat discharge never decreased to less than 80 % of the rise recorded after the first injection of adrenaline. The reduction in size of the discharges was apparently not due to a persistent peripheral vasoconstriction resulting from the early injections of adrenaline, since amyl nitrite inhalation did not consistently increase the amount of sweat expelled (Fig. 2). However, the vasodilator effect of inhaled amyl nitrite may have been limited, for although the dose was larger than that normally administered to humans therapeutically, blood pressure decreased only slightly and heart rate did not increase following inhalation.

An increase in evaporative water loss from the cheek during electrical stimulation of the cervical sympathetic nerve has been observed previously in sheep by Evans (1957) but with only one stimulation in each experiment. In the present experiments up to 4 successive periods of stimulation elicited such a response from the one cheek. Each discharge lasted for only a short time even during prolonged stimulation and none was more than a fraction of the size of an adrenaline-induced or a natural discharge recorded from the trunk. Rebreathing of expired air did not initiate sweating in these experiments although it has been found to do so in man (Bullard, 1964). At the ambient temperatures at which the present experiments were carried out (15–30° C) the effect of rebreathing on sweating might not be

measurable since the effect is only small in man at such temperatures. The sweating mechanisms of man and the sheep might also differ in their response to such conditions.

Allen & Bligh (1969) found that continuous I.V. infusion of adrenaline at rates up to 1·4 μg/kg. min had no effect on the pattern of sweat discharges in Welsh Mountain sheep. The present experiments add to this finding the observation that infusions of both adrenergic and cholinergic drugs are ineffective in producing a continuous discharge of sweat in sheep even at dose rates sufficient to cause considerable changes in blood pressure. When the pulsatile discharges of sweat were blocked by prior administration of phenoxybenzamine there was still no overflow of fluid from the sweat glands although this may have been because the phenoxybenzamine blocked the secretion of sweat into the gland lumen as well as contraction of the myoepithelium around the glands. Although infusions of adrenaline and carbachol did not alter the background rate of cutaneous water loss, they appear to have increased the frequency of sweat discharges at air temperatures of both 10 and 40° C. The increase in frequency of discharges during drug infusion is of uncertain significance since the measurements of frequency were subject to possible sampling errors and because, when carbachol was infused, the effect appeared to persist during the subsequent control period when saline was infused.

A reservation about the findings of the present experiments concerns the extent to which the infusion of adrenaline or the stimulation of a sympathetic nerve trunk is a valid means of simulating natural sympathetic activity. Injection of adrenaline or stimulation of a sympathetic nerve will cause not only a discharge of sweat glands but also vasomotor and other effects. Furthermore, electrical or pharmacological stimulation of the sweating mechanism may exert unphysiological effects on sweat secretion and on myoepithelial contraction of the sweat gland. The variability of response to different modes of sympathetic stimulation is such that on one occasion an intravenous injection of adrenaline elicited an increased rate of evaporative water loss from the cheek immediately after nervous stimulation had been ineffective, and on another occasion a large spontaneous discharge of sweat followed almost immediately after an injection of adrenaline had elicited only a small discharge of sweat from the same skin area. There must be considerable differentiation of function between the fibres in a sympathetic nerve trunk. Indiscriminate electrical stimulation of such a nerve or the intravenous injection of adrenaline are probably little more than crude means of simulating the sympathetic activity that initiates the natural mass discharge of sweat glands in sheep.

I appreciate the guidance of Drs J. Bligh and A. Silver in the preparation of the manuscript, and the technical assistance of Messrs P. Smith and A. J. Barton.

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J. Physiol. (1971), **215**, *pp.* 755–768
With 6 text-figures
Printed in Great Britain

SPINO-SPINAL MECHANISMS IN THE CHLORALOSE ANAESTHETIZED CAT

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(*Received 14 December 1970*)

SUMMARY

1. In chloralose anaesthetized cats the events in the lumbosacral cord that follow stimulation of a forepaw or neck nerve have been examined.

2. Four events take place in the lumbosacral cord after forepaw or neck muscle nerve stimulation. These are: (1) a brief period of generalized enhanced motoneurone excitability, (2) a prolonged and as yet unexplained enhancement of monosynaptic reflexes, (3) a period of presynaptic inhibition in muscle and skin afferent fibres, and (4) a prolonged inhibition of spino-bulbo-spinal reflexes.

3. A variable and sometimes selective reduction of one or another descending effects follows the placing of small lesions in the anterior portion of the suprasylvian gyrus.

INTRODUCTION

The chloralose-anaesthetized cat responds reflexly to almost any form of sensory stimulus by a characteristic jerk. Sometimes studied from a pharmacological standpoint (Hanriot & Richet, 1893; Alvord & Fuortes, 1954; Devanandan, Eccles, Lewis & Stenhouse, 1969*a*, *b*), sometimes mentioned as a curiosity in the course of other investigations (Adrian & Moruzzi, 1939), the neurophysiological events underlying the phenomenon have some unique characteristics. In particular, spino-spinal effects are mediated by the well-known intraspinal pathways (Sherrington & Laslett, 1903; Lloyd, 1942; Lloyd & McIntyre, 1948) but utilize pathways which loop supraspinally and in which cerebral cortex has an important role (Adrian & Moruzzi, 1939; Ascher, Jassik-Gershenfeld & Buser, 1963; Devanandan *et al.* 1969*a*; Abrahams & Falchetto, 1969; Abrahams, 1970*a*). In the present work some of the events that take place in the lumbosacral cord as a consequence of activity in these looping pathways have been examined.

METHODS

All experiments were performed on cats anaesthetized with a single intravenous injection of chloralose (60 mg/kg) following the induction of anaesthesia with ethyl chloride and ether. Body temperature of the cats was controlled at or close to 38° C by a heated table. Blood pressure and respiration were continuously monitored and recorded. Appropriate dorsal and ventral roots of the lumbosacral area were exposed and placed on bipolar platinum electrodes for electrical recording. Nerves of the neck, fore and hind paws were also dissected free and placed on bipolar platinum electrodes which were used for electrical recording and stimulation. All exposed nervous tissue was bathed in a silicone oil (Medical Fluid 360, Dow Corning Silicone) in skin flap baths in the usual manner. Amplification was by a.c. amplifiers including that for the recording of dorsal root potentials (DRPs) when an over-all time constant of 1 sec was used. Records of oscilloscope traces were made photographically.

The technique for testing the excitability of the termination of axons within the spinal cord followed that of Wall (1958). A stainless steel or tungsten micro-electrode (Transidyne type 415–20 or 405–20) was introduced into the spinal cord with a micromanipulator at an angle of about 20° so that the electrode tip would ultimately rest in the ventral horn. This was done either after section of all ventral roots on one side of the cord from L4 to S2, or with all ventral roots except L7 and S1 sectioned. In the latter experiments the L7 and S1 roots were dissected free and a ligature placed round each root so that the roots might be cut during the course of the experiment.

Conduction velocities of fibres in the superficial radial and dorsal interosseus nerves were measured by placing bipolar recording electrodes on the appropriate branch of the brachial plexus proximal to the stimulus site. Because of the short length and rapid conduction velocity of fibres in the biventer cervicis nerve, it was not possible to measure conduction velocity in this way. Records were made from a dorsal rootlet of one of the first three cervical roots at the point of cord entry either by a fine pair of bipolar electrodes placed under the rootlet or by a stainless steel micro-electrode just entered into the rootlet at the point of cord entry.

All brain lesions were placed using subpial suction. Positioning of the lesion was verified by examination of sections cut from the formalin-fixed brain.

RESULTS

L7 direct discharges and segmental reflex excitability changes

As is well known (Alvord & Fuortes, 1954; Abrahams & Falchetto, 1969; Abrahams, 1970*a*), stimulation of a forepaw or neck muscle nerve in the chloralose-anaesthetized cat leads to a discharge in lumbosacral ventral roots after a latency of 12–15 msec. The duration of this discharge is between 8–20 msec and when segmental reflexes are tested during the discharge both monosynaptic and polysynaptic reflexes are found to be greatly enhanced. The monosynaptic reflex facilitation outlasts the duration of direct discharge and may persist for a further 40–60 msec. After the direct discharge polysynaptic reflexes are usually reduced below control levels and may even be abolished (Fig. 1). In a small proportion of animals the direct discharge is weak and the course of interaction in such

experiments is somewhat different. The facilitation of polysynaptic reflexes is minimal, although the monosynaptic reflex still shows a prolonged period of facilitation which commences 25–30 msec after forepaw or neck muscle nerve stimulation.

The effects on polysynaptic reflexes may be examined in reflexes elicited by sural nerve stimulation, or by reflexes recorded from a ventral root

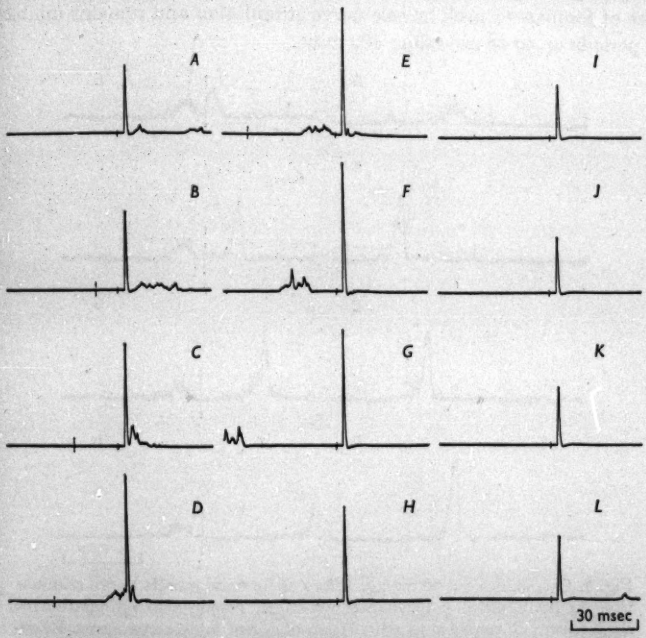

Fig. 1. Cat, chloralose 60 mg/kg. Effect of neck muscle nerve stimulation on lumbosacral reflexes. *A*, control reflex. Effect on reflex of stimulation of biventer cervicis nerve at *B*, 10 msec; *C*, 20 msec; *D*, 30 msec; *E*, 40 msec; *F*, 50 msec; *G*, 80 msec; *H*, 100 msec; *I*, 200 msec; *J*, 300 msec; *K*, 400 msec; *L*, 500 msec before the reflex. Recordings from L7 ventral root. Reflex elicited by posterior tibial nerve stimulation.

one or more segments removed from that showing the presence of monosynaptic reflexes. Typical results of such an experiment are shown in Fig. 2. The polysynaptic reflex vanishes when the interval between conditioning and test stimulus is as brief as 10 msec. When the direct response and the polysynaptic reflex coincide, then the response is enhanced. When

the test reflex occurs late in the direct response it is weakened, and it remains depressed for intervals up to and exceeding 70 msec.

In chloralose anaesthetized cats a third reflex is seen about 10 msec after the end of the main polysynaptic elevation. This is the spino-bulbo-spinal (SBS) reflex (Shimamura & Livingstone, 1963). As previously reported (Abrahams & Falchetto, 1969), this reflex is inhibited within 10 msec of forepaw or neck muscle nerve stimulation and remains inhibited for periods up to or exceeding 400 msec.

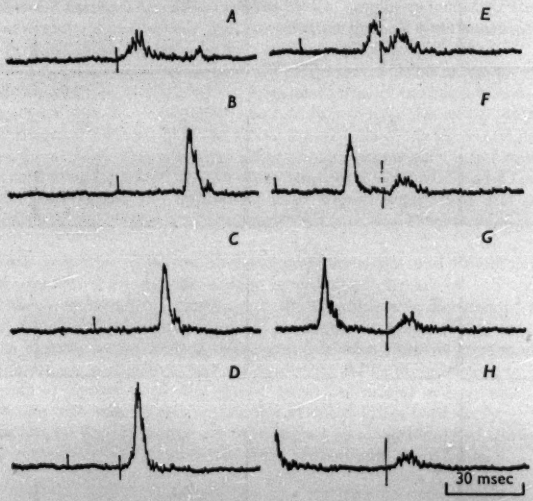

Fig. 2. Cat, chloralose 60 mg/kg. Effect of forepaw muscle nerve stimulation on lumbosacral polysynaptic reflex. *A*, response to stimulation of sural nerve; *B*, response to stimulation of dorsal interosseus nerve. Effect on reflex of stimulation of dorsal interosseus nerve at *C*, 10 msec; *D*, 20 msec; *E*, 30 msec; *F*, 40 msec; *G*, 50 msec; *H*, 70 msec before the reflex. Note inhibition of polysynaptic reflex at short conditioning test intervals, and its reduction at longer intervals. Records from L7 ventral root.

Terminal excitability in the lumbosacral cord in descending interactions

The obvious explanation of the prolonged and selective facilitation of lumbosacral monosynaptic reflexes would be a descending presynaptic action on group Ia fibres. This hypothesis was tested in twenty cats with the method of Wall (1958). No evidence could be found for a presynaptic effect that could be responsible for the prolonged and selective mono-

synaptic facilitation. On the contrary (Fig. 3), evidence has been obtained for a period of depolarization with its peak 40–60 msec after forepaw or neck muscle nerve stimulation. It is possible, with slight modification of the Wall (1958) technique, to simultaneously test terminal excitability and excitability in the orthodromic pathway. With the L7 and S1 ventral roots intact, the electrode was placed 1–2 mm into the cord at the level of

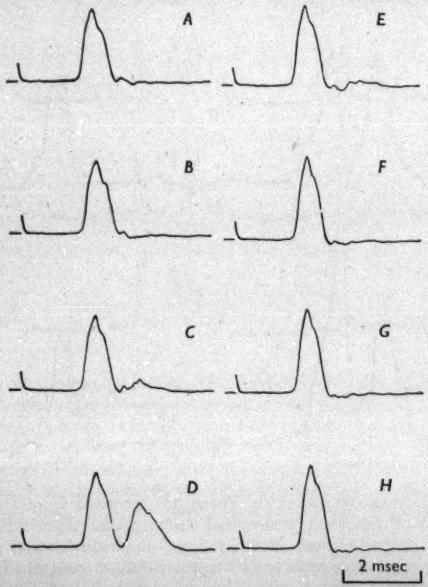

Fig. 3. Cat, chloralose 60 mg/kg. Compound action potentials recorded from lateral gastrocnemius nerve after intraspinal stimulation. Ventral roots L4–6 and S2 sectioned. Micro-electrode about 1 mm into cord at dorsal root entry of L7. Effect of stimulation of neck muscle nerve *B*, 10 msec; *C*, 20 msec; *D*, 25 msec; *E*, 30 msec; *F*, 40 msec; *G*, 50 msec; *H*, 60 msec before intraspinal stimulation.

dorsal root entry and stimulus strength was adjusted so that the compound action potential recorded on the gastrocnemius nerve showed two elevations. With the electrode about 2 mm into the cord the second elevation may be quite small. When the electrode is advanced into the ventral horn, the two elevations are approximately equal (Figs. 3 and 4). The effects of forepaw or neck muscle nerve stimulation were then tested. At the time that the direct response appears on the ventral root, increase in the second

elevation can be seen coincident with the appearance of the direct response (Fig. 3). The early elevation was unaltered at this time, but when the conditioning interval exceeded 40 msec and when the late elevation had returned to normal, the early elevation was enhanced. The early wave is due to antidromic conduction and the late wave due to conduction in the orthodromic pathway, for, following section of the remaining L7 and S1 ventral roots, the early elevation remains intact but the late elevation is lost (Fig. 4).

Fig. 4. Cat, chloralose 60 mg/kg. Potentials recorded from lateral gastrocnemius nerve following intraspinal stimulation. Electrode in ventral horn. Top, control stimuli. Bottom, intraspinal stimulation preceded by stimulus to neck muscle nerve (30 msec). Residual ventral roots (L7 and S1) cut at arrow.

These experiments show that forepaw or neck muscle nerve stimulation leads to an increase in excitability in the orthodromic pathway but this is both early and brief and could be either in motoneurones or interneurones. The experiments also show that there are changes in group Ia terminal excitability following stimulation of forepaw or neck muscle nerves, changes which occur at the time when the prolonged monosynaptic facilitation is present. The changes are in the wrong direction, however, and signal the presence of a period of presynaptic inhibition which should signal a reduction in the monosynaptic reflex, not a facilitation.

Although not immediately relevant to studies of the origin of monosynaptic reflex facilitation, the terminal excitability of cutaneous afferents was also tested in the same experiments by recording from the sural nerve.

The effects of the descending system were found to vary among different cutaneous fibre groups. Further, there were differences in the responses elicited by forequarter muscle or skin nerve stimulation. Terminal depolarization in the fastest cutaneous fibres (velocity 60 m/sec) was found as a late response to forepaw nerve stimulation and persisted for up to 120 msec. Such changes were least easily elicited from stimulation of a mixed nerve (but largely cutaneous nerve) such as the superficial radial and were most

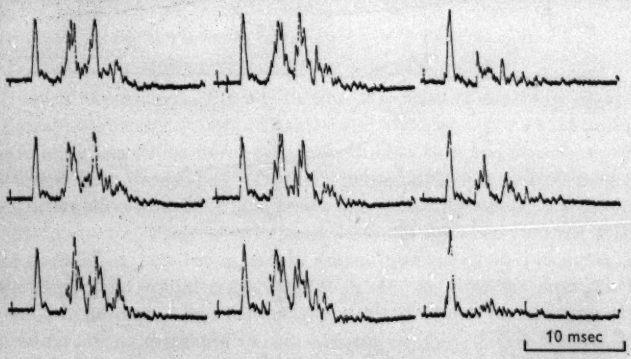

Fig. 5. Cat, chloralose 60 mg/kg. Compound action potentials recorded from sural nerve after intraspinal stimulation. Ventral roots L4 to S2 sectioned. Left panels, control potentials. Middle panels, intraspinal stimulation 30 msec after forequarter nerve stimulation. Right panel, intraspinal stimulation 40 msec after forequarter nerve stimulation. Top records, superficial radial nerve stimulated; middle panel, dorsal interosseus nerve stimulated; bottom panel, biventer cervicis nerve stimulated.

prominent when muscle nerves such as the dorsal interosseus or biventer cervicis were stimulated. When terminal excitability of the slow fibres (15–20 m/sec) was examined there was a period of afferent hyperpolarization, first evident 20–30 msec after a conditioning stimulus and with a duration of 100 msec. This was more readily seen following neck muscle nerve stimulation (Fig. 5).

Dorsal root potentials

The appearance of dorsal root potentials is associated with primary afferent depolarization (presynaptic inhibition) in the spinal cord (Eccles, Magni & Willis, 1962). If the changes in terminal excitability that were found in hind leg group Ia fibres after forepaw nerve stimulation were due to presynaptic inhibition, they should be accompanied by the appearance of dorsal root potentials. In more than thirty experiments such potentials have been consistently recorded from lumbosacral roots after

forepaw or neck muscle nerve stimulation (Fig. 6). The paradoxical relationship between segmental reflex excitability and terminal excitability is detailed in Fig. 6. It can be seen that at a time when Group Ia fibres are apparently inhibited the monosynaptic reflex is still considerably enhanced.

The dorsal root potentials recorded on an L7 and S1 rootlet and which follow forepaw nerve stimulation are of long latency, ranging from 25 to

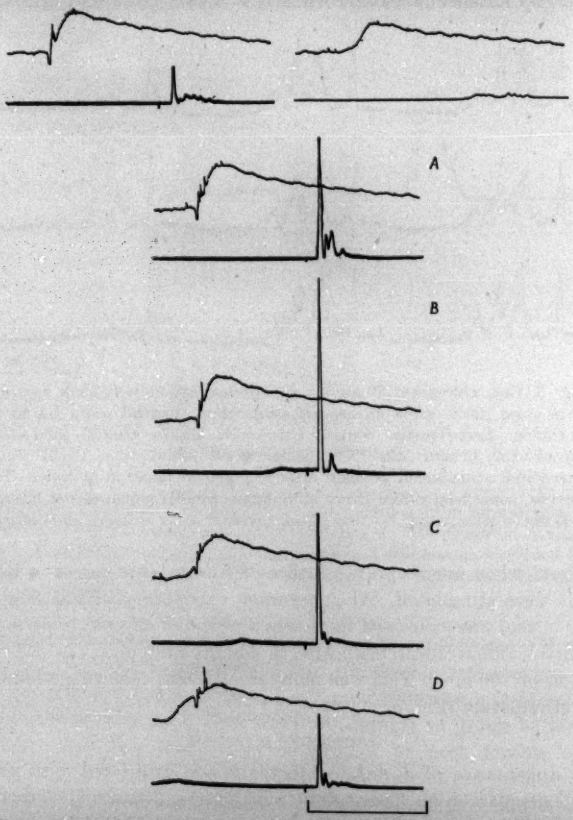

Fig. 6. Cat, chloralose 60 mg/kg. Simultaneous recording of dorsal root potentials and lumbosacral reflexes. Top left, response to posterior tibial nerve stimulation. Top right, response to dorsal interosseus nerve stimulation. Effect of dorsal interosseus nerve stimulation on reflex *A*, 20 msec; *B*, 30 msec; *C*, 40 msec; *D*, 50 msec before reflex. Note time bar represents 12·5 msec for lower (reflex) records, and 50 msec for upper (DRP) records.

40 msec and have their peak some 20 msec or so later. Customarily they have a duration of about 150 msec. They consist only of a long negative nerve DR V (Lloyd & McIntyre, 1949). Following the elicitation of a dorsal root potential from forepaw nerve stimulation, no dorsal root potential can be obtained from stimulation of a hind leg nerve for 2–400 msec and vice versa. The early components of a dorsal root potential elicited from hind leg nerve stimulation DRPs I–IV (Lloyd & McIntyre, 1949) may also be reduced by prior stimulation of a forequarter nerve.

Conduction velocity of fibre groups in forepaw nerves initiating cervico-lumbar reflex interactions

In seven experiments the conduction velocities of the fibre groups in the dorsal interosseus, superficial radial and biventer cervicis that initiated descending interactions were measured. In both skin and muscle nerves it was found that descending effects including SBS loss, direct response, reflex interaction and DRP are present at threshold when only the most rapidly conducting fibres were excited. In the biventer cervicis nerve these fibres conduct at 110–150 m/sec, in dorsal interosseus 90–140 m/sec, and in the superficial radial nerve 80–140 m/sec. As stimulus strength was increased to activate fibres conducting at 50–70 m/sec, the strength of the descending effects increased to a maximum.

Effects of restricted cortical lesions on descending spino-spinal interactions

Evidence has been obtained that the effects of restricted lesions of the frontal cortex are exerted unequally on differing spinal mechanisms. Lesions were made in fourteen cats in the anterior pole of the contralateral suprasylvian and ectosylvian gyrus. Lesions in the region of the anterior syprasylvian gyrus always had some effect on descending effects exerted on the lumbosacral cord. The inhibition of the SBS reflex and monosynaptic reflex facilitation was most readily affected by a small cortical lesion and these effects were almost invariably reduced or abolished by such a lesion. The direct discharge over the ventral roots and its associated brief facilitation of both monosynaptic and polysynaptic reflexes was less affected and may persist even though the later prolonged monosynaptic facilitation was lost. The direct discharge following superficial radial nerve stimulation was least affected by cortical lesions, and often persisted after loss of interactions and direct discharges following biventer cervicis and dorsal interosseus stimulation. The dorsal root potential, although often reduced by cortical lesions, was only rarely abolished. All these effects were associated only with damage to the anterior pole of the suprasylvian gyrus. Lesions of similar size placed more posteriorly in the ectosylvian gyrus were without effect on interactions, direct discharges or dorsal root potentials.

DISCUSSION

In the cat anaesthetized with chloralose, spino-spinal interactions are dependent upon pathways that loop supra-spinally (Adrian & Moruzzi, 1939; Alvord & Fuortes, 1954; Devanandan *et al.* 1969*b*; Abrahams & Falchetto, 1969). The anatomical details of the looping pathway are not known, although there is strong evidence of involvement of the cerebral cortex. Adrian & Moruzzi (1939) found that ablation of the motor cortex eliminated the pyramidal tract discharge that normally follows skin stimulation. Evidence for cortical involvement was also found in an examination of descending spino-spinal systems originating in neck muscle nerves (Abrahams & Falchetto, 1969). In the latter experiments it was found that lesions of the anterior pole of the suprasylvian gyrus, a cortical area receiving input from neck muscles (Landgren & Silfvenius, 1968), abolished descending interactions in acute experiments (Abrahams, 1970*a*). The interactions are not irreversibly abolished and can be demonstrated in animals several days after the placing of a lesion on the anterior suprasylvian gyrus at aseptic operation (Abrahams, 1970*b*).

The cerebral cortex seems able to exert a powerful modulating effect on descending spino-spinal systems, but the cerebral cortex does not appear to be the essential link in an anatomical pathway. In experiments on the role of the cerebral cortex in spinal discharge following sensory stimulus, Ascher *et al.* (1963) found that electrical or chemical stimulation of the appropriate cortical receiving area enhanced the spinal ventral root discharge that follows sensory stimulus. They also found that the topical application of KCl to the appropriate receiving region abolished the ventral root discharge. Despite the loss of response after KCl depression of the cortex, Ascher *et al.* (1963) regard the cortical role as a modulator for they were still able to demonstrate ventral root discharges following ablation of appropriate cortical receiving areas. Further, Buser, St Laurent & Menini (1966) found ventral root discharges following click stimulation to survive removal of the whole neocortex. Our experiments constitute an extension of the findings of Ascher *et al.* (1963) and Buser *et al.* (1966) in that they demonstrate that areas of the cortex are involved in descending spino-spinal interactions. It is also apparent that individual components of the descending interactions may be dependent on the integrity of different cortical areas.

The present experiments, however, have been less concerned with the details of the pathways involved than with the nature of the events that take place in the lumbosacral cord following skin or muscle nerve stimulation in the forequarters. The existence of the chloralose jerk and its electrical analogue, the ventral root discharge, is well known and the intra-

cellular studies of Devanandan *et al.* (1969*a*, *b*) leave little doubt that this discharge is associated with a brief period of EPSP activity in lumbosacral motoneurones. The present experiments have amply confirmed the increased excitability that occurs in this period. A generalized increase in motoneurone excitability could presumably explain the simultaneous facilitation of monosynaptic and polysynaptic reflexes that is seen during the ventral root discharge. The prolonged period of monosynaptic reflex excitability that follows the direct discharge (Alvord & Fuortes, 1954; Abrahams & Falchetto, 1969) is less readily explained. The present experiments fail to show any heightened motoneurone excitability at this time, and indeed the experiments of Devanandan *et al.* (1969*a*) suggest that the reverse happens, for they report that a prolonged period of motoneurone hyperpolarization with decreased synaptic activity follows the EPSP activity. The selective and prolonged monosynaptic reflex facilitation cannot be explained by a period of pre-synaptic hyper-excitability of group Ia fibre terminals. At the time of the prolonged monosynaptic reflex facilitation, evidence from experiments on terminal excitability and from the recording of dorsal root potentials demonstrate a period of terminal depolarization and thus a period of presynaptic inhibition.

Perhaps the simplest explanation of the selective facilitation of the monosynaptic reflex comes from the hypothesis of Frank (1959) and which suggests that a remote (dendritic) facilitation may occur on the dendrite. It seems well established that the majority of synapses including those of Group 1A fibres are not on the motoneurone soma, but are on the motoneurone dendrites (Terzuolo & Llinas, 1965). It is quite possible that descending fibre terminations lie in close proximity to the Ia dendritic terminals and by a local membrane action can modulate the synaptic action of the Ia terminal. This could readily explain the enhanced monosynaptic reflex at a time when the motoneurone is not exhibiting enhanced exictability and the presynaptic fibre is inhibited. It might be expected that the existence of such a system should manifest itself by increased dendritic excitability and thus by increased activity in the orthodromic pathway during intraspinal stimulation. This has not been seen but this too is not unexpected. The dendritic fibres of lumbo-sacral motoneurones are dispersed freely through the cord (Sprague & Ha, 1964). The passage of a current through the stimulating micro-electrode, large enough to excite an appreciable population of dendrites, will simultaneously excite a large population of motoneurones. Activity originating in the dendrites could be masked and thus preclude the ability to distinguish activation of dendritic origin.

No attempt has been made in the present series of experiments to analyse the prolonged inhibition of the SBS reflex, although the existence

of such prolonged effects emphasizes another property of the nervous system of the cat anaesthetized with chloralose. It is a characteristic that responses to sensory stimulation are reduced or eliminated when the stimulus is repeated at brief intervals (Alvord & Fuortes, 1954). The effects on the SBS reflex are in this category and it seems likely that in the cat anaesthetized with chloralose powerful inhibitory systems may be activated by sensory stimuli. The nature of the operation of these systems should itself be of considerable physiological interest.

The present findings suggest that consequences of stimulus application to a cat anaesthetized with chloralose are the near simultaneous activation of a number of what may be independent systems, all with different actions within the spinal cord. There has been some previous attempts to give these phenomena a functional interpretation. Alvord & Fuortes (1954) thought that the mechanism of the chloralose jerk might have some relationship to the mechanisms of the startle reaction. However, it seems likely that following almost any sensory stimulus that the same events take place in the spinal cord, the only obvious differences that occur being in the detail of the timing and the magnitude of the response. Ascher *et al.* (1963) found essentially the same sequence of events in the lumbosacral cord following visual, auditory and cutaneous stimulation. Recently we have found that vestibular nerve stimulation (Abrahams, 1971) leads to a sequence of events in the lumbosacral cord that closely parallels the events that follow stimulation of a forepaw nerve.

It seems likely then that the nervous system of the cat anaesthetized with chloralose is one in which all spinal mechanisms that can be activated by sensory stimulation are nearly simultaneously activated. The response to sensory stimulation is neither appropriate nor graded and normally appears as the well known 'chloralose jerk'. These findings underline the easy access to motor systems that all sensory systems must have, for all sensory systems are inevitably concerned with posture and with movement. Presumably when the response is appropriate and graded it may involve all or only one of the systems described here to an appropriate extent. Direct access to motoneurones (as evidenced by the ventral root discharge) is obviously essential for the direct control of movement. The ability to initiate prolonged periods of inhibition of one or another system is also an obvious asset to the system for it enables irrelevant reflexes to be temporarily suspended. Access to presynaptic or remote systems offers a discrete method of manipulation of individual spinal reflexes, and by specifically being able to manipulate the monosynaptic reflex. It is easy for the body to be stabilized against load fluctuations. The chloralose-anaesthetized animal reveals the existence of a wide range of neurophysiological substrate, which is perhaps the potential armentarium to

sensory inputs whereby they can manipulate the mechanism of posture and movement.

Acknowledgements: Supported by the M.R.C. of Canada. The technical assistance of Miss D. Butler and Miss J. Daynes is gratefully acknowledged.

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With 2 text-figures
Printed in Great Britain

INTESTINAL TRANSFER OF CHOLINE IN RAT AND HAMSTER

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(*Received 18 December 1970*)

SUMMARY

1. The transfer of choline was studied with sacs of everted intestine of rat and hamster.

2. The choline transfer can be divided into two components, a diffusion process and a saturable process. The latter plays a relatively greater part at low concentrations of choline, which include the physiological concentration in the plasma. The saturable process is better seen in the hamster than in the rat.

3. Intestinal transfer of choline is influenced by substances altering the availability of energy in the cell, and by some substances chemically or pharmacologically related to choline. These findings are consistent with some kind of specific mechanism for choline transfer.

4. Part of the choline taken up by the cell appears as a metabolite not yet identified. The formation of the metabolite is a saturable process and is abolished by anaerobic conditions and by homogenization.

5. The results are also discussed in relation to parameters of transfer.

INTRODUCTION

Although choline is a constituent, and possibly an essential one, in the diet, its handling by the intestine has not been extensively studied. Vetper, de la Huerga, Grossman & Popper (1952) found that a third of a 20 mg dose fed to the rat disappeared from the intestine within an hour, while Rohse & Searle (1955) studied disappearance from the ileal loops in dogs. The present results with the rat and hamster intestine were undertaken to supply some information about the mechanisms involved in choline absorption. A preliminary account of the work has been given by Sanford & Smyth (1969).

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METHODS

Experiments were carried out on male white rats bred in the Sheffield Field Laboratories and golden hamsters obtained from J. K. Stewart, Tarvin, Chester. Before experiment the animals were maintained on a commercial diet (Diet 86 Oxoid, London) with food and water freely available.

The preparation was the sac of everted small intestine (Wilson & Wiseman, 1954) as modified by Barry, Matthews & Smyth (1961). When rats were used the combined jejunum and ileum (80–100 cm) was divided into five equal parts and in the present experiments sacs were made from the proximal, middle and distal fifths (sac I, sac III and sac V) representing the proximal jejunum, middle regions of small gut and the distal ileum respectively. In experiments involving the golden hamster the middle 17 cm of the combined jejunum and ileum (19–25 cm) was made into a sac. A few experiments were performed in which this 17 cm was divided to produce two sacs of equal length. These are referred to subsequently as the jejunum and ileum respectively. Bicarbonate saline (Krebs & Henseleit, 1932) was used. The sacs contained initially 1 ml. bicarbonate saline (the serosal fluid), an exception being the smaller sacs which were filled with 0·5 ml. bicarbonate saline. All sacs were incubated in 25 ml. bicarbonate saline for 1 hr at 38° C in equilibrium with a gas phase of 95:5 % oxygen:carbon dioxide unless otherwise stated. Choline in the form of choline chloride was added initially to the mucosal fluid. Other modifications of the mucosal and serosal fluids were made as described in subsequent sections. At the end of the incubation the sacs were quickly rinsed, excess fluid removed on moist filter paper and the fluid transfer determined by weighing, as described by Barry *et al.* (1961). The final mucosal and serosal fluids were collected, the gut homogenized and deproteinized and all three fractions made up to a known volume, and used for the determination of choline or its derivatives as described below. The fractions are referred to as the mucosal fraction, the gut fraction and the serosal fraction respectively.

Transfer parameters. The mucosal transfer is the amount of fluid or solute disappearing from the mucosal fluid, and the serosal transfer the amount appearing in the serosal fluid. The difference between these is the amount retained in the gut wall, provided the substance is neither metabolized nor produced in the gut wall. In the case of fluid all three fractions can be determined gravimetrically. In the case of solutes not metabolized the most accurate measure of mucosal transfer is the sum of the amount retained in the gut wall and the serosal transfer. Although, as shown below, choline is metabolized, the fact that it is estimated by radioactivity enables the same principle to be applied. Hence the mucosal transfer of choline was determined from the radioactivity of the gut fraction and serosal fraction. Choline transfer is expressed as μmole/sac.hr. Mucosal fluid transfer is expressed as ml./sac.hr. Another parameter used is the final concentration ratio, which in this case is the final serosal concentration/initial mucosal concentration. This is a more rigorous criterion of transfer against a concentration gradient than final serosal concentration/final mucosal concentration because it can only be achieved by movement against a gradient and not by metabolism.

Chemical. [Me-^{14}C]choline was obtained from the Radiochemical Centre, Amersham, and determined by liquid scintillation counting using a liquid scintillator for aqueous solutions (Bray, 1960).

Chromatography. Mucosal and serosal fluids and homogenates of the small intestine were analysed to determine whether choline is altered during its passage through the intestinal wall. Samples of these fluids were spotted on Whatman No. 1 chromatography paper and exposed to one of the following solvent systems:

(A) ethyl alcohol:water = 8:1 (descending), (B) *n*-butyl alcohol:ethyl alcohol: water:acetic acid = 8:2:3:1 (ascending), (C) *n*-propyl alcohol:water = 8:1 (descending), (D) *n*-propyl alcohol:formic acid:water = 8:1:1 (ascending), (E) acetone:water:hydrochloric acid = 100:20:1 (ascending). At the end of the run the chromatogram was dried and analysed for radioactivity.

RESULTS

Choline transfer and initial choline concentration

The effect of initial choline concentration on mucosal choline transfer by hamster intestine was studied over a wide range of concentrations from 10 to 7500 μM and the results are seen in Table 1. Plotting the transfers

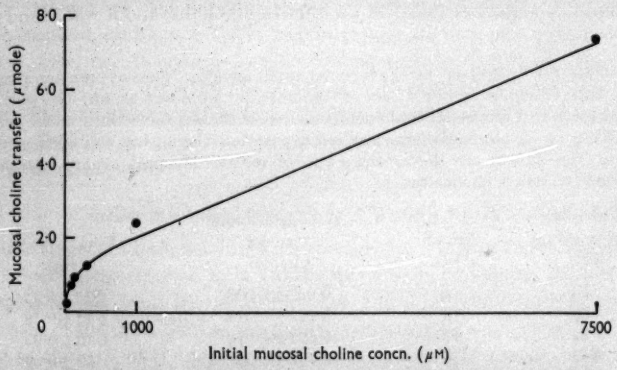

Fig. 1. The transfer of choline from the mucosal fluid (μmole/sac.hr) as a function of the initial mucosal choline concentration. The points represent the observed values. The line represents theoretical values for transfer calculated from (*a*) a saturable process following Michaelis–Menten kinetics ($V = 1{\cdot}6$ μmole/hr, $K = 160$ μM) and (*b*) a diffusion process ($D = 7{\cdot}709 \times 10^{-4}$ μmole/μM).

against the initial concentrations (Fig. 1) suggests that there are two processes involved in choline transfer, (1) a process which is saturable and which dominates the kinetic picture at the lower concentrations and (2) a process which has diffusion kinetics and dominates the picture at high concentrations. If it is assumed that the saturable process follows Michaelis–Menten kinetics, the total rate of transfer will be represented by the equation

$$v = \frac{VC}{C+K} + DC \qquad (1)$$

where v is the total rate, C the mucosal concentration of choline, V the maximum rate of the saturable process, K the affinity constant of the

saturable process and D a diffusion constant. By trial and error values for V, K and D can be found which give a reasonable fit with the experimental values. The line drawn in Fig. 1 has been calculated for $V = 1{\cdot}6$ μmole/hr, $K = 160\,\mu$M and $D = 7{\cdot}709 \times 10^{-4}$ μmole/μM. If these constants can be taken as a reasonable approximation it is now possible from eqn. (1) to calculate the amount of choline transferred by the saturable process and the diffusion process at any particular concentration.

Equation (1) can be tested by using labelled choline and studying its transfer in the presence and absence of a large concentration of unlabelled choline which should competitively inhibit the saturable process but not the diffusion process. Table 1 shows that mucosal transfer of labelled choline at a concentration of 75 μM is 0·574 μmole/hr, and it was found that

TABLE 1. Choline transfer by the hamster small intestine. Sacs of hamster intestine were made from the middle 17 cm of combined jejunum and ileum. The sacs contained initially 1 ml. bicarbonate saline and were shaken for 60 min under aerobic conditions in 25 ml. bicarbonate saline containing choline at the concentration shown. The figures are the means ± S.E. of means. Mucosal choline transfer is expressed as μmole choline/sac.hr

Initial choline concentration (μM)	No. of expts.	Mucosal choline transfer (μmole)	Final concn. ratio
10	5	0·097 ± 0·003	2·17 ± 0·12
25	8	0·226 ± 0·014	2·22 ± 0·18
75	49	0·574 ± 0·012	1·72 ± 0·03
150	5	0·932 ± 0·045	1·53 ± 0·07
300	6	1·285 ± 0·072	1·03 ± 0·07
1000	11	2·429 ± 0·186	0·60 ± 0·03
7500	9	7·417 ± 0·224	0·31 ± 0·03

in the presence of 7425 μM unlabelled choline the rate of transfer was reduced to 0·072 μmole/hr. This supports the view that at least part of the choline is being transferred by a process which can exhibit competitive inhibition. This can be investigated more quantitatively as follows. The K of the saturable process is 160 μM. In the presence of 7425 μM unlabelled choline this should be changed to $160(1+\frac{7425}{160}) = 7585\,\mu$M. If this value is substituted in equation (1) for K instead of 160 μM, it can be calculated that the total rate of transfer is 0·074 μmole/hr, which agrees well with the observed figure of 0·072 μmole/hr.

Movement against a concentration gradient

Table 1 also shows that at concentrations below 300 μM the final serosal concentration is greater than the initial mucosal concentration. This sug-

gests that the saturable process is able to cause movement against a concentration gradient while the other is not. This is consistent with the view that the non-saturable process has the characteristics of diffusion. It should be noted that choline was estimated by radioactivity, and therefore it would be more correct to say that at low initial concentrations of choline the final serosal concentration of choline plus radioactive derivatives produced by metabolism is greater than the initial mucosal concentration. It follows that in experiments to study the saturable process it would be better to use concentrations of choline well below 300 μM, and in fact 75 μM was mostly used.

Some experiments were also carried out with rat intestine. Since this is much larger than the hamster intestine, different regions can be more easily studied, and hence Table 2 gives results for three different parts of the rat intestine. As regards transfer against a concentration gradient only in sac III is there any evidence of a final concentration ratio greater than unity. The rat intestine thus lacks the capacity to transfer choline against a concentration gradient in circumstances where this can be achieved by the hamster intestine.

Variation in choline transfer in different parts of the gut

With the short hamster intestine the regions compared were the upper and lower halves referred to in Table 4 as jejunum and ileum. Table 4 also includes results from the total intestine, and it provides a check on the accuracy of the procedure as the transfers with the total intestine are fairly close to the sum of the jejunal and ileal transfers. The choline transfer is greater in the ileum than the jejunum in contrast to the fluid transfer which is greater in the jejunum. The combined effect of these is to make the final concentration ratio considerably greater in the ileum.

Table 2 shows that in the rat intestine choline transfer is greatest in sac V and least in sac I for concentrations of choline of 75 μM and below.

Effect of various substances on transfer

Apart from non-specific effects on membrane permeability substances affecting transfer may do so by altering the energy supply or affecting some specific site involved in transfer. Substances which are actively transferred and/or metabolized, are likely to change energy availability, and the effects of a number of such substances were studied. A number of other substances related to choline either chemically or pharmacologically were studied with the possibility in mind that these might interfere with sites involved in choline transfer.

TABLE 2. Choline transfer by the rat small intestine. Sacs of rat intestine were made from the upper (sac I), middle (sac III) and lower (sac V) fifths of the combined jejunum and ileum. The sacs contained initially 1 ml. bicarbonate saline with or without 222 mM mannose. The sacs were shaken for 60 min in 25 ml. bicarbonate saline containing choline at the concentration shown. The figures are the means ± S.E. of means. Mucosal choline transfer is expressed as μmole choline/sac.hr

	Sac I			Sac III			Sac V		
Initial choline concentration (μM)	No. of expts.	Mucosal choline transfer (μmole)	Final concn. ratio	No. of expts.	Mucosal choline transfer (μmole)	Final concn. ratio	No. of expts.	Mucosal choline transfer (μmole)	Final concn. ratio
(*a*) No mannose present									
10	4	0·032 ± 0·004	0·55 ± 0·06	5	0·068 ± 0·010	1·22 ± 0·13	4	0·088 ± 0·003	0·91 ± 0·06
25	14	0·091 ± 0·003	0·60 ± 0·03	18	0·156 ± 0·005	1·02 ± 0·03	13	0·261 ± 0·009	0·96 ± 0·04
75	6	0·247 ± 0·010	0·55 ± 0·02	5	0·389 ± 0·025	0·84 ± 0·04	6	0·642 ± 0·027	0·83 ± 0·05
7500	6	8·657 ± 0·176	0·28 ± 0·01	6	7·405 ± 0·420	0·24 ± 0·01	7	6·050 ± 0·303	0·11 ± 0·01
(*b*) Mannose present									
25	12	0·170 ± 0·008	0·88 ± 0·03	15	0·250 ± 0·008	1·23 ± 0·09	7	0·273 ± 0·006	0·75 ± 0·04
7500	3	7·759 ± 0·302	0·15 ± 0·01	3	7·159 ± 0·417	0·12 ± 0·02	3	7·578 ± 0·420	0·11 ± 0·01

Effect of metabolized hexose

Table 2 includes experiments with rat intestine both in the absence and presence of mannose, with choline concentrations of 25 μM and 7500 μM. At 25 μM choline the presence of mannose causes stimulation of choline transfer by 87, 60 and 5% respectively in sacs I, III and V. At 7500 μM choline the presence of mannose does not cause any large change in choline transfer.

It is of some interest to compare the effects of mannose on fluid transfer and choline transfer, and further details of these experiments are shown in Table 3, which shows also how the transfer from the mucosal fluid is divided between the gut wall and the serosal fluid. In sacs I and III, mannose increases fluid transfer by 227 and 202% respectively, and choline by only 87 and 60%, but in spite of this the final concentration ratio of choline is increased. In sac V the stimulation of choline transfer is very small, and the final concentration ratio falls. Another fact of some interest is the very high concentration of choline in the gut wall, due to the different fractions of fluid and choline retained in the gut wall. For example in sac I in the absence of mannose, 0·60 ml. fluid and 0·091 μmole choline leave the mucosal compartment and of this, 65 and 80% are retained in the gut wall. In the presence of mannose the fractions of fluid and choline retained in the gut wall are 36 and 71% respectively. Thus much more of the extra choline transfer stimulated by mannose is retained than of the extra fluid and this results in a very high concentration in the gut wall (64·6 μM) compared with the serosal fluid (22·0 μM). In sac V an even greater fraction of the choline leaving the mucosal fluid remains in the gut wall, so that a very high concentration in the gut wall is produced. These findings will be discussed later in relation to selection of parameters for transfer.

Table 5 includes experiments on hamster intestine with either glucose or mannose, and shows that mannose did not stimulate choline transfer, while glucose caused some inhibition. Both glucose and mannose caused some stimulation of fluid transfer.

Effect of transferred substances

As a representative of a transferable hexose α-methylglucoside was used, and as a representative of an amino acid L-methionine. The effect of these on hamster intestine is seen in Table 5. When choline is initially present in a concentration of 75 μM, methionine in a concentration of 7·5 mM reduces choline transfer by 27%, and at 28 mM by 35%. At these concentrations α-methylglucoside reduces choline transfer by 30 and 46% respectively. They also reduce the final concentration ratio of choline. At 7500 μM

TABLE 3. Effect of mannose on transfer of fluid and choline by the rat intestine. These are the experiments included in Table 2, where the initial mucosal concentration of choline was 25 μM. Mannose when present was initially in the serosal fluid. The figures are the means ± S.E. of means. Fluid transfer is expressed as ml./sac.hr and choline transfer as μmole/sac.hr

			Fluid			Choline			Final choline concentration		
Region	Mannose concn. (mM)	No. of expts.	Mucosal transfer (ml.)	Gut uptake (ml.)	Serosal transfer (ml.)	Mucosal transfer (μmole)	Gut uptake (μmole)	Serosal transfer (μmole)	Serosal fluid (μM)	Gut wall (μM)	Final concn. ratio
Sac I	0	13	0·60 ± 0·03	0·39 ± 0·02	0·21 ± 0·04	0·091 ± 0·003	0·073 ± 0·003	0·018 ± 0·001	15·0 ± 0·7	50·0 ± 2·0	0·60 ± 0·03
	222	12	1·96 ± 0·12	0·70 ± 0·06	1·26 ± 0·07	0·170 ± 0·008	0·120 ± 0·006	0·050 ± 0·003	22·0 ± 0·9	64·6 ± 2·7	0·88 ± 0·03
Sac III	0	18	0·81 ± 0·03	0·44 ± 0·02	0·37 ± 0·04	0·156 ± 0·005	0·121 ± 0·004	0·035 ± 0·001	25·5 ± 0·6	85·2 ± 3·7	1·02 ± 0·03
	222	15	2·45 ± 0·09	0·72 ± 0·05	1·73 ± 0·12	0·250 ± 0·008	0·159 ± 0·005	0·091 ± 0·005	33·0 ± 0·9	91·9 ± 4·3	1·32 ± 0·09
Sac V	0	13	1·19 ± 0·09	0·57 ± 0·03	0·62 ± 0·08	0·261 ± 0·009	0·221 ± 0·007	0·040 ± 0·003	24·1 ± 1·0	137·9 ± 4·5	0·96 ± 0·04
	222	7	2·47 ± 0·10	0·74 ± 0·03	1·73 ± 0·12	0·273 ± 0·006	0·222 ± 0·005	0·051 ± 0·003	18·8 ± 1·0	133·0 ± 5·1	0·75 ± 0·04

TABLE 4. Choline transfer by the hamster small intestine. Sacs of hamster intestine were made from the middle 17 cm, or from the proximal (jejunal) or distal (ileal) halves of this. The sacs contained initially 1 ml. (whole) or 0·5 ml. (jejunum and ileum) bicarbonate saline and were shaken for 60 min under aerobic conditions in 25 ml. bicarbonate saline containing 75 μM choline. The figures are the means ± S.E. of means. Choline is expressed as μmole/sac.hr and mucosal fluid transfer as ml./sac.hr

Region	No. of expts.	Mucosal fluid transfer (ml.)	Serosal choline transfer (μmole)	Gut choline (μmole)	Mucosal choline transfer (μmole)	Final concn. ratio
Whole	49	1·02 ± 0·03	0·220 ± 0·004	0·354 ± 0·008	0·574 ± 0·012	1·72 ± 0·03
Jejunum	10	0·52 ± 0·04	0·099 ± 0·007	0·155 ± 0·007	0·254 ± 0·011	1·47 ± 0·06
Ileum	10	0·34 ± 0·02	0·107 ± 0·004	0·207 ± 0·011	0·314 ± 0·014	1·97 ± 0·08

TABLE 5. Effect of sugars and amino acid on choline transfer. Experimental conditions are as in Table 1 with substances added to the mucosal and serosal fluids as described below. The figures are the means ± S.E. of means. Mucosal choline transfer is expressed as μmole/sac.hr and mucosal fluid transfer as ml./sac.hr

Initial choline concn. (μM)	Substance added to		No. of expts.	Mucosal fluid transfer (ml.)	Mucosal choline transfer (μmole)	Final concn. ratio
	Mucosal fluid	Serosal fluid				
75	—	—	49	1·02 ± 0·03	0·574 ± 0·012	1·72 ± 0·03
75	7·5 mM mannitol	—	5	0·92 ± 0·11	0·511 ± 0·036	1·51 ± 0·07
75	28 mM mannitol	—	7	0·53 ± 0·09	0·454 ± 0·031	1·51 ± 0·06
75	7·5 mM methionine	—	4	1·21 ± 0·09	0·419 ± 0·018	1·24 ± 0·10
75	28 mM methionine	—	4	0·90 ± 0·03	0·371 ± 0·020	1·19 ± 0·03
75	7·5 mM α-methylglucoside	—	6	1·62 ± 0·07	0·402 ± 0·015	1·19 ± 0·03
75	28 mM α-methylglucoside	—	7	1·21 ± 0·04	0·308 ± 0·016	1·03 ± 0·05
75	28 mM glucose	—	7	1·99 ± 0·16	0·440 ± 0·019	1·05 ± 0·03
75	—	222 mM mannose	6	1·94 ± 0·09	0·595 ± 0·026	1·38 ± 0·05
7500	—	—	9	0·81 ± 0·07	7·417 ± 0·224	0·31 ± 0·03
7500	28 mM mannitol	—	4	0·43 ± 0·01	6·613 ± 0·588	0·32 ± 0·03
7500	28 mM methionine	—	4	0·68 ± 0·08	7·015 ± 0·129	0·33 ± 0·04
7500	28 mM α-methylglucoside	—	5	1·24 ± 0·06	7·107 ± 0·611	0·24 ± 0·02

choline, the effects of methionine and α-methylglucoside are negligible. The effect of L-methionine and α-methylglucoside on fluid transfer is much less and on the whole stimulates fluid transfer. Mannitol of similar molecular weight to the hexoses and amino acid used, was selected as an osmotic control. While causing reduction in fluid transfer mannitol had much less effect on choline transfer than α-methylglucoside or L-methionine.

Effects of substances related to choline

As a specific transfer mechanism is more obvious in the hamster than the rat, these experiments were confined to hamster intestine. The results are seen in Table 6. Some of these substances have a large inhibitory effect on both fluid transfer and on choline transfer. Inhibition of choline was only considered specific in those cases where the reduction in choline transfer was very much greater than that of fluid transfer. The only substance which can be regarded as a specific inhibitor of choline transfer is hemicholinium-3 although tetraethylammonium chloride did cause greater inhibition of choline than of fluid transfer. Carbachol and hexamethonium bromide had little effect at the concentrations used, while decamethonium iodide (7·5 mM) and physostigmine salicylate (7·5 mM) both caused non-specific inhibition of transfer. The result with physostigmine salicylate (1 mM) is interesting in that it caused little reduction in fluid transfer or appearance of choline in the gut wall, but reduced serosal choline transfer by 48%.

Conditions affecting choline transfer

Table 7 shows the results of experiments in which a number of different conditions were tested on choline transfer in hamster intestine. As might be expected a fall in temperature, anaerobic conditions and 2×10^{-4} M 2,4-dinitrophenol caused a marked reduction in choline transfer. The insensitivity of the choline transfer system to Na^+ is shown by the small reduction in mucosal choline transfer when NaCl was replaced by mannitol. Under these conditions fluid transfer was almost abolished.

Of interest also is the reduction in transport caused by 10^{-3} M ouabain in the absence of K^+ while 10^{-3} M ouabain or the absence of K^+ alone had little effect.

Chromatographic analysis

Analysis of the serosal fluid in experiments where sacs of hamster intestine were incubated with [^{14}C]choline (75 μM) showed the existence of two peaks. Separation of these was obtained best with ethyl alcohol : water = 8:1 (descending) and the results reported here apply to analysis with this system. Fig. 2 shows these two peaks, and superimposes on this the analysis of the initial mucosal fluid. It is evident that one of the peaks in the serosal fluid is choline, while the other must be some metabolite formed

TABLE 6. Effect of chemically and pharmacologically related substances on choline transfer. Conditions are as in Table 1 with 75 μM choline present initially in the mucosal fluid. Related substances were present initially in the mucosal fluid as described below. The figures are the means ± S.E. of means. Mucosal and serosal choline transfer are expressed as μmole/sac.hr and mucosal fluid transfer as ml./sac.hr

Additions	No. of expts.	Mucosal fluid transfer (ml.)	Inhibition (%)	Serosal choline transfer (μmole)	Inhibition (%)	Mucosal choline transfer (μmole)	Inhibition (%)	Final concn. ratio
None	49	1·02 ± 0·03	—	0·220 ± 0·004	—	0·574 ± 0·012	—	1·72 ± 0·03
7·5 mM tetraethylammonium chloride	7	0·81 ± 0·08	21	0·120 ± 0·006	46	0·342 ± 0·018	40	1·02 ± 0·04
1 mM carbachol	2	0·82	20	0·186	16	0·500	13	1·60
1 mM decamethonium iodide	4	0·74 ± 0·06	28	0·177 ± 0·008	20	0·510 ± 0·012	11	1·66 ± 0·02
7·5 mM decamethonium iodide	4	0·43 ± 0·04	58	0·079 ± 0·004	64	0·273 ± 0·017	52	0·85 ± 0·05
1 mM physostigmine salicylate	6	0·91 ± 0·05	11	0·111 ± 0·005	50	0·432 ± 0·018	25	0·92 ± 0·04
7·5 mM physostigmine salicylate	4	0·40 ± 0·05	61	0·042 ± 0·003	81	0·139 ± 0·001	76	0·46 ± 0·04
75 μM hemicholinium-3	3	0·88 ± 0·07	14	0·173 ± 0·002	21	0·510 ± 0·007	11	1·49 ± 0·06
1 mM hemicholinium-3	5	0·79 ± 0·11	23	0·069 ± 0·007	69	0·197 ± 0·013	66	0·64 ± 0·05
1 mM hexamethonium bromide	4	0·86 ± 0·08	16	0·211 ± 0·010	4	0·572 ± 0·027	0	1·71 ± 0·05
7·5 mM hexamethonium bromide	3	0·68 ± 0·04	33	0·200 ± 0·014	9	0·536 ± 0·029	7	1·78 ± 0·13

from choline. It has already been pointed out that the concentrations of total radioactivity are greater in the serosal than in the mucosal fluid, but since there is a metabolite of choline present, it is not possible to say that choline moves against its concentration gradient. The analysis can be carried further by examining the radioactivity in the initial mucosal fluid, in the final mucosal and serosal fluids, and comparing the amount of radioactivity due to choline and the amount due to the metabolite. The results are shown in Table 8 and it is evident from this that choline appears in the

TABLE 7. Effect of various conditions on choline transfer by hamster intestine. The conditions are as in Table 1 with alterations as described below. The initial mucosal choline concentration was 75 μM. The figures are the means ± S.E. of means. Mucosal choline transfer is expressed as μmole choline/sac.hr and mucosal fluid transfer as ml./sac.hr

Conditions	No. of expts.	Mucosal fluid transfer (ml.)	Mucosal choline transfer (μmole)	Final concn. ratio
Aerobic control	49	1·02 ± 0·03	0·574 ± 0·012	1·72 ± 0·03
Temperature = 28° C	6	0·74 ± 0·08	0·378 ± 0·017	0·84 ± 0·05
2×10^{-4} M 2,4-dinitrophenol	5	0·04 ± 0·03	0·158 ± 0·010	0·66 ± 0·03
Absence of K^+ in medium	7	0·84 ± 0·04	0·512 ± 0·018	1·63 ± 0·07
10^{-3} M ouabain in serosal fluid	8	0·70 ± 0·07	0·518 ± 0·016	1·59 ± 0·05
Absence of K^+ in medium and 10^{-3} M ouabain in serosal fluid	7	0·43 ± 0·05	0·327 ± 0·029	1·30 ± 0·11
Replacement of NaCl by mannitol	5	0·10 ± 0·03	0·408 ± 0·027	1·94 ± 0·15
Anaerobic conditions	6	0·04 ± 0·05	0·134 ± 0·003	0·73 ± 0·05

serosal fluid in about the same concentration as in the mucosal fluid, while the metabolite appears at a much higher concentration in the serosal fluid than in the mucosal fluid. The sum total of radioactive substances is much greater in the final serosal fluid than in the initial or final mucosal fluid. While the Table shows a very high final serosal concentration/final mucosal concentration ratio for the choline metabolite it should be remembered that this is partly explained by the very different volumes of mucosal and serosal fluid. In fact the amount of metabolite entering the serosal fluid is only about twice that entering the mucosal fluid.

It has been suggested that part of the choline transfer is due to a saturable process, and it is therefore of interest to see whether the formation of a metabolite is also a saturable process. Some information on this can be obtained by studying the percentage of radioactivity in the choline peak

and in the metabolite peak when different initial concentrations of choline were present. This is shown in Table 9 where it is seen that the higher the concentration of choline the smaller the percentage of radioactivity accounted for by the metabolite. The amount of metabolite formed is therefore not proportional to the initial choline concentration and there is some evidence for the existence of a saturable process involved either in the

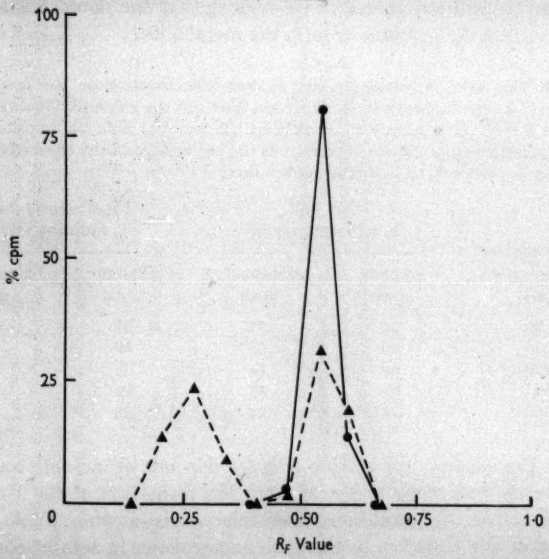

Fig. 2. Chromatographic analysis of the initial mucosal fluid (●—●) and final serosal fluid (▲---▲) from experiments where sacs of hamster intestine were incubated with [^{14}C]choline (75 μM). Samples were spotted on Whatman No. 1 paper and exposed to ethyl alcohol:water = 8:1 (descending chromatography). The points represent the percentage of the total counts on the chromatogram observed at different R_F values.

TABLE 8. Distribution of choline and its metabolite in the final serosal and mucosal fluid. Conditions are as in Table 1 with 75 μM choline present initially in the mucosal fluid. The amount and concentration of the choline metabolite are expressed in units of choline

	Choline + metabolite		Choline		Metabolite	
	Amount (μmole)	Concn. (μM)	Amount (μmole)	Concn. (μM)	Amount (μmole)	Concn. (μM)
Initial mucosal fluid	1·875	75·0	1·875	75·0	—	—
Final mucosal fluid	1·319	54·8	1·266	52·6	0·053	2·2
Final serosal fluid	0·192	115·7	0·090	54·1	0·102	61·6

formation of the metabolite or in its transfer to the serosal fluid. It will be noted that in the gut wall in some cases the choline and its metabolite recovered did not account for all the radioactivity present.

Experiments were carried out to determine whether the amount of metabolite formed was dependent on the region of the intestine used. The results with hamster intestine are shown in Table 10 and with rat intestine in Table 11. In both animals it is evident that the ileum has a greater capacity than the jejunum to form the metabolite.

TABLE 9. The effect of initial mucosal choline concentration on the proportion of choline and its metabolite in the final serosal fluid and the gut wall. Conditions are as in Table 1 with choline present initially in the mucosal fluid as described below. Choline and its metabolite are expressed as the percentage of the total radioactivity appearing in the gut wall and final serosal fluid

Initial mucosal choline concn. (μM)	Gut wall % radioactivity in		Final serosal fluid % radioactivity in	
	Choline peak	Metabolite peak	Choline peak	Metabolite peak
25	33	57	31	69
75	43	55	50	50
300	56	44	63	37
1000	65	35	69	31
7500	76	24	88	12

Since the capacity for choline transfer depends on aerobic conditions, experiments were done to see whether the formation of the metabolite depended on aerobic conditions. That this is so is shown in Table 12. Also included in this Table are results with homogenates in aerobic conditions. This shows that almost no metabolite is found in these conditions, indicating that the formation of the metabolite depends on some structural relationship destroyed by homogenization.

Effect of luminal contents on choline metabolism

To test whether the metabolite produced was due to luminal contents hamster intestine was ligated at both ends and removed from the animal's body. The intestinal contents were then washed directly into an incubating flash with 25 ml. 75 μM choline chloride and incubated under aerobic conditions for 1 hr at 38° C. Subsequent analysis of this fluid showed that no metabolite was formed.

TABLE 10. Choline transfer by the hamster small intestine. Sacs were made from the proximal and distal halves of the middle 17 cm of the combined jejunum and ileum. Conditions were as in Table 3 with 75 μM choline present initially in the mucosal fluid. The choline transferred from the mucosal fluid has been analysed chromatographically to determine the amounts appearing as choline and as choline metabolite. The results are expressed as μmole choline/sac.hr

	Gut wall			Serosal transfer			
Region	Metabolite (μmole)	Choline (μmole)	% as metabolite	Metabolite (μmole)	Choline (μmole)	% as metabolite	Total % as metabolite
Jejunum	0·061	0·093	37	0·024	0·077	24	32
Ileum	0·146	0·054	69	0·072	0·037	66	68

TABLE 11. Choline transfer by the rat small intestine. Sacs were made from the upper (sac I), middle (sac III) and lower (sac V) fifths of the combined jejunum and ileum. The experimental conditions were as in Table 2 with 25 μM choline present initially in the mucosal fluid. The choline transferred from the mucosal fluid has been analysed chromatographically to determine the amounts appearing as choline and as the choline metabolite. The results are expressed as μmole choline/sac.hr

	Gut wall			Serosal transfer			Total		
Sac	Metabolite (μmole)	Choline (μmole)	% as metabolite	Metabolite (μmole)	Choline (μmole)	% as metabolite	Metabolite (μmole)	Choline (μmole)	% as metabolite
I	0·010	0·068	13	0·001	0·016	6	0·011	0·084	12
III	0·016	0·073	14	0·003	0·027	10	0·019	0·100	13
V	0·193	0·041	80	0·013	0·017	43	0·206	0·058	86

TABLE 12. The influence of various conditions on the production of the choline metabolite by the hamster small intestine. For aerobic and anaerobic sac experiments the conditions were as in Table 5 except that the final serosal fluid and intestinal wall were pooled and analysed chromatographically. When the effect of homogenizing the intestine was studied, 2g intestine in a final volume of 50 ml. bicarbonate saline containing 75 μM choline was used, this being incubated under aerobic conditions for 1 hr at 38° C. At the end of the experiment this fluid was analysed for choline and its metabolite

Condition	% total radioactivity in Metabolite peak	% total radioactivity in Choline peak
Aerobic sac	54	42
Anaerobic sac	5	91
Aerobic homogenate	2	98

DISCUSSION

When choline is initially present in the mucosal fluid of the everted sac preparation of rat or hamster intestine it is taken up by the cells and transferred to the serosal fluid. Part of the choline appears as such and part in the form of a metabolite not yet identified. This happens in both rat and hamster, but some features of the process are best seen in the hamster. If the total amount of choline and its metabolite are combined and considered together, transfer against a concentration gradient can be shown, and this is better seen in the hamster than in the rat. In both animals, in the absence of a metabolizable hexose, the lower part of the small intestine has more capacity for handling choline than the upper part. The uptake can be described in terms of a saturable process and a diffusion process, and at high concentrations the transfer process is characterized much more by the diffusion component. Precise information is not available about the physiological concentration of choline in the intestinal lumen, but the following considerations would suggest that the experiments reported here cover the physiological range. The plasma concentration in man, cat and dog is approximately 10 μM (Bligh, 1952). The choline daily allowance of the rat and of the hamster has been given as 40–80 mg and 640 mg/day.kg body wt. respectively (Spektor, 1956). These would be equivalent to a transfer rate of about 0·9 μmole choline/sac.hr for the rat and 19 μmole choline/sac.hr for the hamster. In addition there is also probably a considerable enterohepatic circulation of choline, as choline is present in substantial amounts in the bile. It thus seems likely that the physiological uptake of choline by the intestine involves some kind of mechanism in addition to diffusion.

The effects of various substances and conditions supports this con-

clusion. Newey & Smyth (1964) showed that the transfer of amino acid is stimulated by glucose and inhibited by galactose, effects which they attributed to increase or decrease in the amount of energy available for the amino acid transfer mechanism. In the present experiments, choline transfer in rat was stimulated by mannose in sac I and III but very little in sac V. The results in sac I and III are consistent with the concept of provision of extra energy for transport processes by metabolized hexoses. The smaller effect in sac V needs more consideration. Barry *et al.* (1961) showed that in sac V glucose caused less stimulation of fluid transfer than in the other sacs. This could be due to (*a*) limitation of entry of glucose into the epithelial cell, (*b*) the fact that the transfer process was already nearly saturated in the absence of glucose, or (*c*) inability of the cell to metabolize glucose. A reduction in hexose metabolism was demonstrated by Newey, Sanford & Smyth (1970) when glucose was present initially in the serosal fluid, and in these conditions hexose metabolism was not limited by the entry mechanism at the luminal membrane. The failure of mannose to stimulate choline in sac V could therefore be due to the reduced metabolism of hexose in sac V, or alternatively, to the fact that the choline transfer mechanism is nearly saturated in the absence of glucose. The fact that fluid transfer is stimulated more than choline transfer may indicate a topographical arrangement in the cell whereby some transport processes may be more favourably situated than others for available energy.

In the hamster intestine mannose did not stimulate choline transfer and glucose caused some inhibition. This is in agreement with the results of Hardcastle, Newey & Smyth (1968) who found that glucose inhibited amino acid transfer in the hamster, and this they attributed to the fact that on account of its low rate of metabolism glucose was using more energy by its transfer than it was contributing by metabolism. This is consistent with the difference between glucose and mannose reported here, as mannose is not actively transferred (Barry, Eggenten & Smyth (1969) and therefore does not use energy. The inhibition of choline transfer by methionine and α-methylglucoside, substances which are transported but not metabolized, is also in keeping with this concept.

The fact that choline transfer is inhibited by anaerobic conditions, 2,4-dinitrophenol and a reduction in temperature support the concept of a mechanism for choline transfer dependent on metabolic energy. The continuation of choline transfer when the Na^+ concentration is reduced to 25 m-equiv/l. suggests a relative insensitivity of the transfer system to Na^+. Ouabain causes some inhibition, but only when K^+ is absent from the medium, a condition which in the rat inhibits other transfer systems normally ouabain insensitive (Newey, Sanford & Smyth, 1968).

Specific transfer mechanisms usually involve specific sites for attachment of the substance transferred, and this was tested by using a number of substances related chemically or pharmacologically to choline. A number of these inhibited choline transfer but inhibited fluid transfer as well, and could not therefore be regarded as competing for a transfer site. The only substance giving evidence of specific inhibition was hemicholium-3, although tetraethylammonium chloride caused greater inhibition of choline transfer than of fluid transfer.

The formation of a metabolite from choline is a matter of considerable interest. Its formation is a saturable process, being seen best at concentrations where transfer against a gradient is observed. This raises the question whether the formation of the metabolite is part of the transfer mechanism. Table 8 shows that the final serosal/final mucosal concentration ratio is much greater for the metabolite than for choline itself, and might suggest that formation of the metabolite may be part of the mechanism for choline transfer. However, it is important to recognize that a high concentration in the serosal fluid of a substance produced in the gut wall does not necessarily indicate an active transport mechanism, a point which has been discussed in detail by Newey *et al.* (1970). Nevertheless, the amount of metabolite in the serosal fluid is twice that in the mucosal fluid in spite of the much more favourable conditions for diffusion toward the mucosal side. Certainly if the sum of choline and its metabolite is considered, there is some reason to postulate an active transport mechanism. Another interesting feature of the metabolite formation is that it is an aerobic process and also requires some cellular organization which is destroyed by homogenization.

Other authors have observed chemical transformations in choline in the intestinal lumen. Wünsche (1940) showed that trimethylamine could be produced by intestinal bacteria, while Norris & Benoit (1945) in extending this work showed that trimethylamine oxide was excreted in greater amounts when choline was fed than when injected intraperitoneally, indicating that the intestine was involved in the conversion. De la Huerga & Popper (1951) have suggested that bacteria destroy most of the intestinal choline. While the metabolite described in the experiments has not been identified, it is not trimethylamine. It is also not formed in the mucosal fluid, as when contents of the hamster small intestine were collected and incubated with choline, none could be detected. This does not eliminate the possibility that it could be formed by the bacteria of the mucus or glycocalyx, as these may not be removed by the technique used for preparing the sacs.

Another substance produced from choline in the intestine is phosphorylcholine, which is produced in acetone extracts of the intestine of calf and

rabbit (Wittenberg & Kornberg, 1953). This also seems unlikely to be the metabolite described here, but until the metabolite has been identified, speculation on its nature is not very useful.

Although *in vitro* studies on the intestine have been in progress for about 20 years there are still discrepancies in the interpretation of experiments due to different parameters used for assessing transfer. In every *in vitro* study it is therefore useful to examine critically different parameters in order to see if different conclusions could be drawn from them. Two kinds of parameters are used, (1) involving transfer rates and (2) involving concentrations. Among the former is the mucosal transfer, i.e. the amount leaving the mucosal fluid and we have always maintained that this is the most reliable parameter for assessing transfer. This is particularly so for substances not metabolized where the mucosal transfer can be measured by the sum of the serosal transfer and uptake in the gut wall. To use either of these two separately as assessment of transfer could be fallacious as the results obtained here clearly show that the distribution of mucosal transfer between gut uptake and serosal transfer varies greatly in different conditions, and is affected particularly by the rate of fluid transfer.

The unreliability of using concentration parameters only is illustrated by the effect of mannose on choline transfer. In sac V mannose has little effect on mucosal choline transfer, but stimulates serosal transfer. However, mannose produces a greater stimulation of fluid transfer and as a result causes a lower concentration of choline both in the gut wall and serosal fluid. Thus a conclusion about the effect of mannose on choline transfer based only on concentrations might be erroneous. Another interesting point emerges from the effects of mannose in sac I and III. It is possible for a substance to stimulate transfer of fluid more than that of a solute and yet produce a better final concentration of that solute either in the serosal fluid or in the gut wall. The very simple explanation is that there is initially a substantial volume of fluid in these compartments but none of the solute in question. Another point of interest is the difference between the concentration of choline in the gut wall and serosal fluid. This is due to the fact that a greater proportion of choline transferred remains in the gut wall than that proportion of fluid transferred. This suggests that the achievement of equilibrium between gut wall and serosal fluid is a slow process in relation to the duration of the experiment. It raises serious doubts about the assumption made in determining intracellular concentrations that the extracellular fluid in the gut wall is in equilibrium with the surrounding medium.

We are indebted to Miss Lynne Ashmore for valuable technical assistance. We are also indebted to the Medical Research Council and to John Wyeth and Brother and Leo Laboratories Limited for financial support.

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J. Physiol. (1971), **215**, *pp.* 789–804
With 6 text-figures
Printed in Great Britain

THE REFLEX NATURE OF THE PRESSOR RESPONSE TO MUSCULAR EXERCISE

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(*Received 21 December 1970*)

SUMMARY

1. In anaesthetized cats tetanic contraction of the hind-limb muscles, elicited by stimulating the ventral roots L6–S1, caused a rise of arterial blood pressure, usually accompanied by small increases in heart rate and pulmonary ventilation: in decerebrate cats, all components of the response were much increased.

2. With tetani of different strengths, obtained by stimulating with different intensities at the same frequency, the pressor response increased with increasing tension.

3. When muscle contraction had been abolished by gallamine, or when dorsal roots L6–S1 had been sectioned, ventral root stimulation no longer caused a pressor response. The response is therefore a reflex, initiated in the exercising limb.

4. The pressor response was not affected by section of all articular nerves to knee and ankle joints, or by section of the vagi. The stimulus therefore originates in the contracting muscles alone.

5. The pressor response is potentiated by occluding the circulation through the working muscles. Reasons are discussed for concluding that the stimulus is chemical rather than mechanical, and that the 'metabolic receptors' for this exercise reflex are the free endings of group III and IV sensory nerve fibres located around the blood vessels.

INTRODUCTION

One of the main unsolved problems in exercise physiology is the nature of the stimulus giving rise to the cardiovascular and respiratory responses that muscular exercise produces. It has been known since the turn of the century that muscular activity is accompanied by an increase in arterial

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blood pressure (Bowen, 1904; Krogh & Lindhard, 1913) and it was originally postulated that the pressor response is due to a direct action of the motor cortex on the 'circulatory centres' (Krogh & Lindhard, 1913; Gillespie, 1924). This idea did not find experimental support, however, and an alternative suggestion was put forward that the pressor response is reflexly elicited by afferent fibres originating in the exercising muscle (Patterson, 1928). This hypothesis has since gained considerable strength from the work of Alam & Smirk (1937, 1938) and Asmussen, Nielsen & Wieth-Pedersen (1943). More recently, Asmussen & Nielsen (1964) and Lind, Taylor, Humphreys, Kennelly & Donald (1964) have shown that the rise in arterial blood pressure accompanying muscular contractions is potentiated when the circulation through the working muscles is occluded.

Though this work on human subjects provides suggestive, indirect evidence, direct experimental proof of the reflex nature of the pressor effect has not been obtained. It has seemed that animal experiments would have to be performed in order to establish unequivocally the reflex nature of the pressor response, and to study its characteristics in more detail. So far, however, experiments using animals have not confirmed the observations on man. In fact, only the opposite effects, i.e. depressor responses, have been described (McDowall, 1936; Euler & Liljestrand, 1947). In these experiments, the hind-limb muscles were stimulated directly in a way which would have led indiscriminately to stimulation, and subsequent blockade, of a large number of afferent fibres from the muscles, which could well have distorted the effect of any appropriate afferent input arising in the contracting muscles themselves. The possibility of artifacts such as these was avoided in the present experiments; for the muscles were activated by ventral root stimulation. As reported in a preliminary communication, muscular contractions then produce a reflex pressor response (Coote, Hilton & Perez-Gonzalez, 1969). As the small myelinated and unmyelinated afferent fibres in muscle can produce similar cardiovascular effects (Coote & Perez-Gonzalez, 1970), they are probably the sensory fibres which initiate the circulatory response to exercise in the experimental animal and in man.

METHODS

Experiments were performed on cats which were anaesthetized with I.V. chloralose (70–75 mg/kg) or decerebrated at the intercollicular level, after preliminary induction of anaesthesia with ethyl chloride and ether. Laminectomy was then performed to expose the lumbar and sacral segments L4–S1.

The triceps surae muscle was prepared for recording tension and a thread was tied round its tendon. The position of this marker thread in relation to a drill inserted into the lower end of the tibia was measured for an ankle flexion of 90 °; this was the muscle length in all experiments. The tendon was then detached from the calcaneum.

Arterial blood pressure was recorded from either the left carotid artery or femoral artery via a saline-filled polyethylene cannula connected to a pressure transducer (Bell and Howell Ltd., Consolidated Electrodynamics). The amplified pressure-wave form was also used to trigger a tachograph unit (Devices Ltd) to give a record of heart rate. Respiration was monitored as air-flow, using a low-volume pressure transducer (Grass Instruments Ltd, PT5A) connected to a tracheal cannula. The output of all recording instruments was amplified and displayed on a 4-channel pen recorder (Devices Ltd).

In a few experiments the venous outflow from the triceps surae was recorded via a photoelectric drop counter (Devices Ltd), the procedures for isolating the muscle outflow being similar to those described by Hilton (1953). In these experiments heparin (1000 i.u./kg) was given I.V. at the end of all surgical procedures.

For recording, the animal was fixed in a stainless-steel frame and suspended in the prone position by means of pins, two of which were inserted bilaterally into the iliac crest, and two in the vertebrae above the level of laminectomy. A paraffin pool was made after the skin and remaining layer of vertebral muscles had been sewn to a brass ring. To expose the cord the dura was incised lengthwise. The ventral roots, L6, L7 and S1 on the right side, were carefully dissected, sectioned close to the cord and placed on stainless-steel stimulating electrodes. Contraction of hind-limb muscles was elicited by electrical stimulation of these ventral roots using square-wave pulses 0·1 msec in duration at 25–50 Hz via an RF isolation unit (Grass, SIU 478A) from a stimulator (Grass, S8). For isometric recording of contractions of the triceps surae muscle the hind limb was fixed at the knee and ankle to prevent movement and the triceps surae tendon was attached to a copper–beryllium bar on which was mounted a pair of strain-gauges. These were so arranged that tension applied to the bar caused distortion of the gauges, which formed one half of a wheatstone bridge, the output of which was displayed on the pen recorder.

RESULTS

Tetanic contractions of the hind-limb muscles, elicited by stimulation of the ventral roots L6, L7 and S1 at frequencies of 25 and 50 Hz for 45–60 sec, always caused a rise of arterial blood pressure (Fig. 1).

Characteristics of the blood pressure response

Anaesthetized preparations. The time to onset of the pressor response from the beginning of the contraction period was measured in sixty-four tests carried out in four different experiments. It was estimated as the time in which the systolic blood pressure had risen by 5 mm Hg above control levels during a maximal sustained tetanus of the hind-limb muscles. The mean value was 2·5 (S.E. $\pm$ 0·19) sec. The arterial blood pressure usually reached a steady level which was maintained throughout the rest of the contraction period, as shown in Fig. 1(*c*), the maximal rise of systolic pressure being around 55 mm Hg (40% above control). In some cases, particularly in the lower ranges of stimulus intensities, the arterial blood pressure rose continuously during the period of muscle contraction (Fig. 1*a*, *b*). Immediately after the tetanus the blood pressure fell steeply

to a level slightly above control values and then returned gradually to normal, usually within 1–1·5 min after the end of stimulation. The above records were taken from tests in which the tetani were submaximal. With tetani in response to supra-maximal stimulation (see Figs. 4, 5), the pressor response diminished as the tension began to decline, though following the latter after a variable delay. In most experiments these pressor effects were accompanied by small increases in heart rate ($< 5\%$) and in rate and depth of respiration. Pupillary dilatation was occasionally observed.

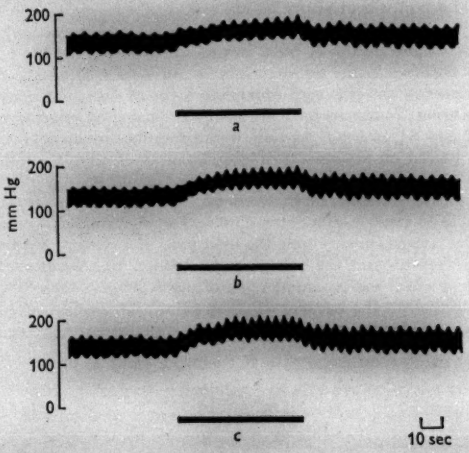

Fig. 1. Cat, chloralose. Records of arterial blood pressure showing effects of tetanic contractions of the right hind-limb muscles of different magnitudes elicited by stimulation at different strengths of the peripheral cut ends of the ventral roots L 6–S 1 at a frequency of 50 Hz for 60 sec. Ventral roots stimulated at $\times 2$ motor threshold (*a*), $\times 4$ (*b*), and $\times 5$ (*c*).

In three experiments the levels of arterial blood pressure at rest were almost the same and remained steady during the course of the whole experiment. In these the question was investigated whether there is any correlation between the magnitude of the pressor response and the strength of the contraction which caused it. The tension developed by the triceps surae muscle was used as a sample of that produced in all the leg muscles, which were contracting simultaneously. To vary strength of contraction the ventral roots were stimulated at a fixed frequency of 50 Hz but with different intensities. The magnitude of the pressor response was expressed as a percentage increase above the resting level. The relationship between these responses and the peak tension developed by the muscle is illustrated

in Fig. 2. The extent to which the response increased with tension (plotted on a logarithmic scale) is indicated by the calculated regression line.

Decerebrate preparations. The pressor responses to tetanic contraction of hind-limb muscles in decerebrate cats were qualitatively very similar to those obtained in anaesthetized animals, but they were more pronounced. Maximal increases of 75 mm Hg were recorded, which is some 80% above the resting systolic levels. Similarly, the accompanying changes in heart rate and respiration were more marked and consistent. These are illustrated in Fig. 3. Increases in heart rate ranging from 14 to 20% were recorded in these experiments, together with marked increases in rate or

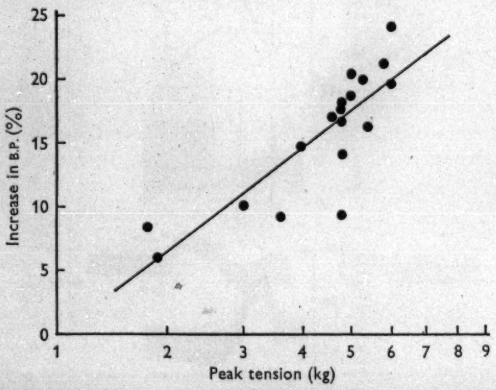

Fig. 2. Relationship between pressor responses to tetanic contractions of hind-limb muscles and tension developed by one group of these muscles (triceps surae) in three experiments. Ordinate, increases in arterial blood pressure expressed as percentages above resting level: abscissa, peak tension developed by triceps surae. Calculated regression line has been drawn.

depth of respiration, and sometimes in both. Two out of the five cats in which decerebration was carried out displayed movement of the jaw, extrusion of claws, pupillary dilatation and piloerection. A post-mortem examination showed that the level of section in these cats was rostral to the intercollicular line and even spared part of the posterior hypothalamus. Another feature of these decerebrate preparations was that on cessation of the tetanic contractions the arterial blood pressure fell steeply to a level below that before stimulation. This post-stimulation vasodepressor effect was often accompanied by a bradycardia.

In three of these cats, blocking the action of the vagus with atropine sulphate (0·5 mg/kg, I.V.) did not alter the pressor, heart rate or respira-

tory responses during contractions, but post-stimulation bradycardia no longer occurred.

Neurogenic origin of the response

These responses are caused by muscular contractions; for when contractions are abolished by gallamine triethiodide (Flaxedil, 3 mg/kg I.V.) ventral root stimulation produces no change of arterial blood pressure, heart rate or respiration.

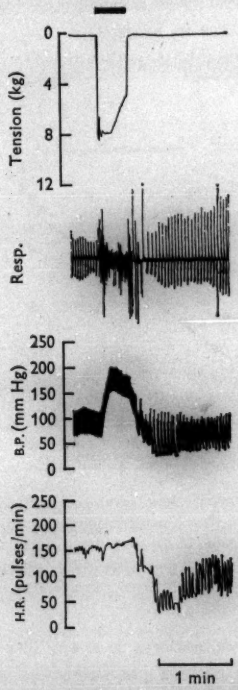

Fig. 3. Decerebrate cat. Records (from above downwards) of tension developed by triceps surae, respiration (Resp.), arterial blood pressure (B.P.) and heart rate (H.R.). At signal, tetanic contraction of the right hind-limb muscles elicited by stimulating peripheral cut ends of ventral roots L 6–S 1, at 0·3 V and 50 Hz. Both common carotid arteries tied.

The response is, in fact, reflex in origin, the stimulus arising in the exercising limb. When the sensory input to the spinal cord from the contracting hind-limb muscles was interrupted by section of the dorsal roots L 6, L 7 and S 1, one by one, on the stimulated side, the size of the pressor

response progressively decreased: it was abolished when all three roots had been cut (Fig. 4). In other experiments the dorsal roots were crushed close to the dorsal root ganglion: this was equally effective in abolishing the responses.

It was unlikely that this reflex originated in joint receptors, as had been suggested for the respiratory response by Comroe & Schmidt (1943), since, in the present experiments, the limb was fixed in a way that precluded

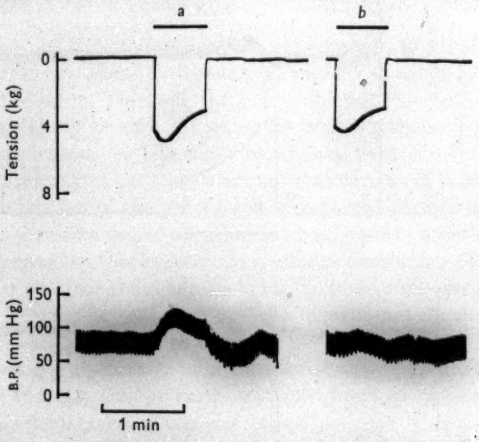

Fig. 4. Cat, chloralose. Records of arterial blood pressure and tension developed by triceps surae. At signals, tetanic contractions of the right hind-limb muscles elicited by stimulating peripheral cut ends of ventral roots L 6–S 1 at 0·4 V and 50 Hz, before (*a*) and after (*b*) section of dorsal roots L 6–S 1.

joint movement. Moreover, this possibility was examined specifically in three experiments in which the articular nerves supplying the knee, and the peroneal, tibial and sural nerves supplying ankle and foot, were sectioned close to the joints. Sectioning these nerves had no effect on the responses subsequently elicited, so a reflex from joint receptors can be excluded. The possible participation of lung inflation afferents was excluded by sectioning both vagi (three experiments), which had no effect on the circulatory responses.

Effect on the responses of restricted blood flow to the contracting muscles

The results of the previous section show that the stimulus for the cardiovascular and respiratory responses arises within the contracting

muscles themselves. This stimulus could be mechanical or chemical. Evidence in favour of the latter was provided by the early experiments of Alam & Smirk (1938) on man, who found that occlusion of the circulation through the contracting muscles potentiated the response. Both Alam & Smirk (1938) and Lind, McNicol & Donald (1966) showed further that the response persisted beyond the period of muscular contraction and until the occlusion was relieved.

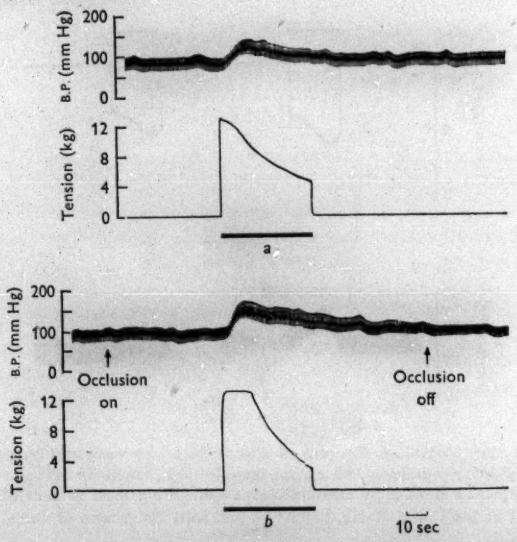

Fig. 5. Cat, chloralose. Records of arterial blood pressure and tension developed by triceps surae. At signal, tetanic contractions of hind-limb muscles elicited by stimulating peripheral cut ends of ventral roots L6–S1 for 45 sec at 50 Hz, before (*a*) and after (*b*) occlusion of right iliac artery (indicated by arrows).

The effect of vascular occlusion was therefore tested in the present experiments. For this purpose, a loose loop of surgical thread was placed around the iliac artery in the abdomen and passed through a mid-line incision in the abdominal wall and left protruding after the abdomen was closed. In each experiment a series of tetanic contractions of increasing strength was elicited and the maximal pressure response was determined. A tetanus slightly stronger than that yielding a maximal response was then used to elicit the control pressor response. The iliac artery was occluded and a similar supramaximal contraction was elicited 1 min after

the beginning of occlusion. Fig. 5 shows a typical result of such a test. The response to contraction developing a peak tension of 12·8 kg with normal circulation is shown above (*a*), and that to a contraction developing 13·0 kg tension during arterial occlusion is shown below (*b*). The pressor response was greater in *b*, where the mean arterial blood pressure rose from 90 to 150 mm Hg (65 %), than in *a*, where the rise was from 90 to 125 mm Hg (40 %). In addition, the rate of rise of pressure was increased during arterial occlusion, the values being 4 mm Hg/sec for *a* and 8 mm Hg/sec for *b*.

There was, in this and most such experiments, a slightly increased tension during occlusion, though the parameters of ventral root stimulation were the same. This was the reason for using control contractions which were already supramaximal for the pressor response produced with unrestricted circulation. The slight increase in tension developed by the muscle with impaired circulation cannot therefore have been responsible for the potentiation of the pressor reflex response. Further evidence supporting this occlusion was obtained in an experiment in which submaximal tetani were used. In this experiment a tetanus developing 1 kg tension in triceps surae was just about threshold for the pressor responses, producing only a 5 % increase in mean pressure with unrestricted circulation. Partial occlusion of the circulation to the limb for as little as 25 sec during the tetanus produced an increase in mean arterial blood pressure of 25 %. The blood flow through triceps surae was monitored at the same time to confirm the effectiveness of the occlusion.

In most experiments occlusion was maintained after muscle contraction had ceased. The arterial blood pressure then did start to fall at the end of contraction, but it fell more slowly than in the tests carried out with free circulation, a further fall to the control level occurring when the occlusion was released (also shown in Fig. 5).

Relationship between local hyperaemic response and strength of muscle contraction

It is well known that, when skeletal muscles contract, a pronounced, local functional hyperaemia occurs. In view of the possibility that the chemical cause of this phenomenon is the same as that initiating the reflex pressor response, the relationship was examined between varying strengths of contraction of triceps surae, as had been achieved above by ventral root stimulation at 50 Hz with different intensities, and the extent of the resulting functional hyperaemia in this muscle.

In plotting the results shown in the graphs of Fig. 6 (three experiments), the changes in conductance were first calculated by dividing blood flow (ml./min) by mean arterial pressure (mm Hg) at different times during the

tests. The resting levels were almost the same in each experiment and remained steady during the whole experiment: the increase in conductance, expressed as a percentage increase above the resting level in each experiment, has been plotted against peak tension of triceps surae in graph (*a*). If there is a real, functional relationship between resistance

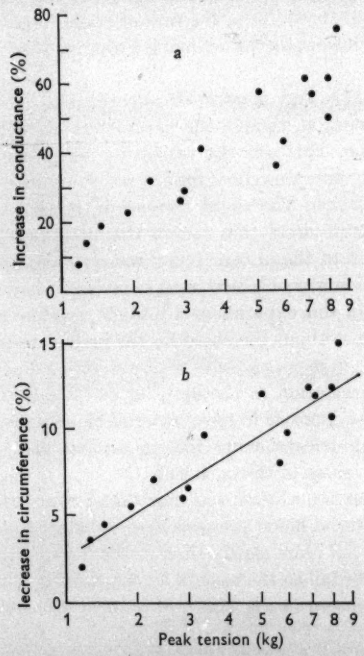

Fig. 6. Relationship between the local hyperaemic response and strength of muscle contraction in triceps surae (three experiments). *a*, ordinate, increase in conductance (flow/pressure) in triceps surae, expressed as percentage above resting level: abscissa, peak tension developed by triceps surae. *b*, ordinate, increase in resistance vessel circumference ($\sqrt[4]{}$(conductance)) in triceps surae, expressed as percentage above resting level: abscissa as in *a*: calculated regression line has been drawn.

vessel size and the concentration of a chemical product of contraction, some measure should be sought corresponding to fibre length in the walls of resistance vessels. As an approach to this ideal, the conductance figures have been used to calculate values related to 'resistance vessel circumference'. This was achieved by taking the fourth root of the calculated con-

ductance, both resting and during tests, and expressing each increase as a percentage above the resting level in each experiment. These increases have then been plotted against peak tension of triceps surae (graph *b*). As in the case of the pressor responses to contraction (Fig. 2), the extent to which 'vessel circumference' increased with tension (plotted on a logarithmic scale) is indicated by a calculated regression line.

DISCUSSION

The present investigation has shown that muscle contraction in the cat gives rise to a reflex rise in arterial blood pressure. This is similar to the reflex pressor response to sustained hand-grip in man described by Lind *et al.* (1964) and analysed by Lind *et al.* (1966). Indeed, the suggestion that the pressor response to muscular exercise in man is dependent on a reflex from the exercising muscles is quite an old one (Patterson, 1928; Alam & Smirk, 1937). One possible reason contributing to the fact that it has not gained much favour among physiologists is the lack so far of a suitable model from animal experiments. The early attempts of McDowall (1936) and Euler & Liljestrand (1947) to provide one seemed, on the contrary, to show opposite effects; for they found falls of arterial blood pressure to accompany contraction of hind-limb muscles in anaesthetized animals. These apparently contradictory findings can now be explained by the different methods used to bring about muscular contraction. In these early experiments direct electrical stimulation of the hind-limb muscles was used, a procedure which would have stimulated many afferent fibres and could have blocked transmission in them subsequently. Moreover, the low-threshold afferent fibres are those from mechanoreceptor nerve endings which are known to cause, when stimulated all together in this way, a reflex fall of blood pressure. This, therefore, would be the primary effect of the stimulation used. Further, any afferent discharge originating in the contracting muscles subsequently would be distorted in quite an unpredictable way. In the present experiments the muscles were made to contract by stimulating the appropriate ventral roots, thus leaving the afferent supply from the muscles intact and avoiding any such artifacts. Under these conditions a pressor response was invariably obtained, accompanied by tachycardia and an increase in rate and depth of respiration. The anaesthetic may have had some depressant effect on this response; for it was much larger when the experiment was repeated in decerebrate preparations.

Lind *et al.* (1966) felt sure that the response in man was reflex; for when they occluded the circulation in the exercising arm, thus preventing any possible chemical mediator from leaving the limb in the venous blood, the

response was, if anything, enhanced. More recently, Lind, McNicol, Bruce, Macdonald & Donald (1968) came very close to demonstrating the existence of the reflex mechanism in humans when they showed that the pressor response could not be evoked in a patient with syringomyelia when he performed hand grips on the affected side. Lind *et al.* (1966) had emphasized that the pressor responses were not associated with pain; for discomfort did not arise from sustained hand-grip contractions until about half-way through a contraction held to fatigue. Similarly, Lewis (1942) and Dorpat (1952) stated that the earliest onset of pain during sustained contractions occurred after 20–25 sec. In the present experiments the arterial blood pressure began to rise 2·5 sec after the beginning of contraction, and frequently reached peak value after 15 sec. Extrapolating from the work in man it is reasonable to conclude that the responses took place in the absence of an afferent input that would have given rise to pain.

In the present investigation, the reflex nature and origin of the response could be proved; for, if muscle contraction was abolished by gallamine, or the appropriate dorsal roots had been sectioned, ventral root stimulation had no effect on blood pressure. On the other hand, after cutting all articular nerves the responses were unimpaired, so they must have originated in the contracting muscles themselves. A further possibility that had to be considered is that the primary reflex response to muscular contraction is respiratory, and that increased ventilation led secondarily to the rise of blood pressure and heart rate through the enhanced activity of lung inflation receptors. This was tested by cutting both vagi, after which the cardiovascular responses to muscle contraction were unchanged.

The next question to be considered therefore was that of the nature of the receptors within the muscles that were initiating the reflex cardiovascular and respiratory responses to exercise. From the work in man it had seemed possible that the receptors were responding to some material produced during contraction, since circulatory occlusion usually enhanced the pressor response to muscle activity (Alam & Smirk, 1938; Staunton, Taylor & Donald, 1964). In our experiments too, occluding the arterial inflow to the hind limb was found to increase the magnitude and rate of rise of the pressor response to contraction. As is well known, the blood flow through the triceps muscle is reduced during tetanic contractions, due to mechanical compression of the intramuscular vessels, and this reduction of flow is naturally greater with increasing strengths of contraction. Similar results were obtained by Humphreys & Lind (1963) in human subjects. It is apparent that artificial occlusion of the circulation only exaggerates that which commonly occurs normally during a tetanic contraction.

In experiments without occlusion it was shown that the magnitude of

the pressor response was proportional to the tension developed by the triceps surae muscle during isometric contractions. Tension was recorded in this muscle as a sample of the activity in the rest of the hind-limb musculature. There will have been differences in absolute tension developed by the various limb muscles, but it is likely that the strength of stimulation required to elicit a maximal contraction in the triceps surae would also cause most of the hind-limb muscles to contract maximally. Therefore, it is reasonable to conclude that the pressor responses were proportional to the strength of the contractions in the whole active muscle group. A similar relationship has been found in experiments on human subjects (Gillespie, 1924; Patterson, 1928; Alam & Smirk, 1937; Lind *et al.* 1964). These findings raise the question whether the stimulus arising in the contracting muscles might not be entirely chemical. The main mechanoreceptor afferent fibres arise from muscle spindles or Golgi tendon organs; the former are unloaded during contractions in which the stimulus to the ventral roots is subthreshold for the γ-efferent fibres (as they were in the major part of our experiments), and the firing rate of the latter is not increased and may even be decreased during the relatively short periods of occlusion used in the present experiments (Perez-Gonzalez, 1969). Moreover, Coote & Perez-Gonzalez (1970) have shown that the group I and group II somatic afferent fibres do not reflexly excite any part of the sympathetic output, whereas group III and group IV afferent fibres do so. As might be expected, therefore, studies of the activity of identified afferent fibres, during contractions of a strength to elicit pressor responses, have not demonstrated firing patterns appropriate to sensory fibres producing the response in any of the known mechanoreceptor afferents, nor in non-mechanoreceptor afferent fibres conducting at rates above 17 m/sec (Perez-Gonzalez, 1969). This observation correlates well with the finding of Coote & Perez-Gonzalez (1970) that only somatic afferent fibres conducting at rates below 15 m/sec reflexly activate sympathetic efferent pathways. The possibility can therefore be excluded that mechanoreceptor endings initiate the cardiovascular reflex, either by a direct response to a mechanical stimulus or by a modification of their mechanical response by a chemical mediator, as was suggested by Dejours (1962) when considering the initiation of the reflex respiratory response to muscular contraction.

The most likely conclusion, therefore, is that the receptors concerned are connected to the group III and possibly group IV afferent fibres which have been shown to be involved in pressor reflexes, and that they are metabolic receptors stimulated by a chemical substance (or substances) released in the muscles during activity. It seems preferable to use the term 'metabolic receptors' rather than 'peripheral chemoreceptors' as the word 'chemoreceptor', has for so long been associated with the central receptors

specifically responsive to a low P_{O_2} (and to some extent to a raised P_{CO_2} and a lowered pH). Kao (1963), who showed that the reflex respiratory response in dogs could be elicited by muscle contraction brought about by ventral root stimulation, also demonstrated in cross-circulation experiments, in which the hind-limb muscles of the recipient were those giving rise to the reflex response, that rendering the donor dog hypoxic or hypercapnic did not induce any change in ventilation in the recipient animal. It is so likely that both the cardiovascular and respiratory responses are part of the same reflex, initiated by the same receptors, that these together may be properly called the exercise reflex.

There are numerous small myelinated and unmyelinated afferent fibres in muscle which have only been regarded so far as pain fibres but which could function as metabolic receptors. These fibres are connected to free nerve endings, many of which are located around blood vessels in the muscle (Lim, Liu, Guzman & Braun, 1962; Barker, Ip & Adal, 1962) and which therefore are ideally distributed to respond to accumulation within the interstitial fluid of some metabolite. Some of these small afferent fibres have already been shown to respond to non-nociceptive stimuli, such as occlusion of the artery to a muscle for only a few seconds (Iggo, 1961). The possibility that they could subserve a non-nociceptive function, such as to initiate cardiovascular and respiratory reflexes during contraction, does not exclude their participation in the pain mechanism. Indeed, according to recent theories of pain perception (Goody, 1957; Melzack & Wall, 1965) the small myelinated muscle afferents would be expected to play a non-nociceptive role even if they could signal pain under certain circumstances.

When judging the extent of the local dilatation of resistance vessels in contracting muscles in relation to the strength of contraction, it is usual to use rate of blood flow as a measure of the vascular response. In fact, however, the measure actually required is the length of the smooth muscle fibres in the vessel walls. As an approximation, in this investigation, 'conductance' was first calculated from flow and perfusion pressure (in order to take account of changes in the latter) and the fourth root of this was used as an index of change of smooth fibre length. Comparison of the graphs of Figs. 2 and 6(*b*) then show that the size of the pressor response has much the same relationship to the tension developed by the contracting muscles as has the extent of the local dilatations of resistance vessels in these muscles (as judged by the change in 'vessel circumference'). This would be consistent with the suggestion of a real relationship, i.e. that the local hyperaemic response and the reflex pressor (or exercise reflex) response are the consequence of similar processes within the muscle and are initiated by the same chemical stimulus.

This work was supported in part by a grant to J.F.P.-G. from the Wellcome Trust.

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J. Physiol. (1971), **215**, *pp.* 805–817
With 6 text-figures
Printed in Great Britain

ACTION POTENTIALS AND RELEASE OF NEUROHYPOPHYSIAL HORMONES *IN VITRO*

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(*Received 29 December 1970*)

SUMMARY

1. Isolated rat neurohypophyses were studied *in vitro* and the hormones released on electrical stimulation of the pituitary stalk or on exposure to excess potassium were estimated by a milk-ejection assay.

2. The stalk was stimulated with trains of 500 stimuli, or multiples thereof, applied at different frequencies. Below frequencies of *ca.* 35 c/s, hormone release was found to depend on the total number of stimuli applied as well as on the frequency of stimulation. Above *ca.* 35 c/s, identical numbers of stimuli were progressively less effective as the frequency of stimulation was increased, and the dependence of the hormone output on the total number of stimuli was less apparent.

3. The amplitude of the compound action potential recorded from the neurohypophysis following electrical stimulation of the stalk was found to decrease as a function of the frequency of stimulation. Stimulation at 50 c/s reduced its amplitude about sevenfold within 30 sec.

4. The addition of tetrodotoxin (TTX) to the incubation media abolished the compound action potential recorded from the neural lobe as well as the release of hormones evoked by electrical stimulation. Resting release, however, was unaffected by TTX.

5. In TTX-treated neural lobes, excess potassium was still effective in eliciting graded secretory responses. This indicates the independence of the release process from the action potential generating mechanism and suggests that TTX-paralysed preparations represent a useful model for the study of hormone release in the absence of conducted action potentials.

6. The release of hormones from the neurohypophysis and the release of neurotransmitters at chemical synapses both depend on the entry of calcium into the nerve terminals following their depolarization by invading

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action potentials. In both systems, experimental separation of the release mechanism can be achieved by the use of TTX. These and other parallels suggest that the release process is similar.

INTRODUCTION

The demonstration by Douglas & Poisner (1964*a*) that the release of vasopressin from the nerve endings of the hypothalamo-neurohypophysial tract can readily be studied *in vitro* has led to rapid progress in the understanding of mechanisms involved in the release of neurohypophysial hormones. Preparations of rat isolated neural lobe liberate vasopressin on electrical stimulation of the pituitary stalk or on exposure of the nerve endings to excess potassium. The process is dependent on the presence of external calcium and is inhibited by external magnesium; these findings have been confirmed repeatedly (Haller, Sachs, Sperelakis & Share, 1965; Daniel & Lederis, 1967) and have been extended to the release of oxytocin (Dicker, 1966; Ishida, 1968). The release of vasopressin is accompanied by calcium uptake by the neurosecretory terminals (Douglas & Poisner, 1964*b*).

From these results one may conclude that the entry of calcium into the nerve endings is a key event of 'stimulus-secretion coupling' and is presumably caused by a transient decrease of the resting potential of the nerve fibre terminals. Increased concentrations of external potassium apparently depolarize the terminals directly, since potassium can release hormones in the absence of external sodium which would be required for the propagation of impulses along the hypothalamo-neurohypophysial tract. The mechanism of action of electrical stimuli applied to the pituitary stalk is less clear: theoretically, they could passively invade the secretory terminals, or they might cause membrane depolarization by setting up propagated action potentials.

Evidence is presented below showing that electrical stimuli applied to the pituitary stalk *in vitro* generate action potentials and that these elicit hormone release from the neural lobe. A quantitative study has been made of the influence of several parameters of electrical stimulation on hormone output, in particular of train duration, total number of stimuli applied and frequency of stimulation. Further, we demonstrate that the release mechanism can be separated experimentally from the impulse generating machinery by the use of the puffer-fish poison, tetrodotoxin (TTX).

The results support the view of Douglas (1968) that the mechanisms for release of hormones from the neurohypophysis and for the release of neurotransmitters from chemical synapses are essentially similar.

METHODS

Hormone release evoked by electrical stimulation of the pituitary stalk. Neurohypophyses were removed, following decapitation, from eighty-four adult rats (Carworth strain, 200–300 g body wt.) of both sexes. The cut end of the pituitary stalk was tied to a platinum wire electrode and each preparation immersed in a small test-tube containing 1·5 ml. of modified 'Locke solution' (NaCl 154 mM, KCl 5·6 mM, $CaCl_2$ 2·2 mM, $MgCl_2$, 1·0 mM, $NaHCO_3$,6·0 mM, glucose 10 mM), maintained at 37 °C and gassed with 95 % O_2/5 % CO_2. The preparations were preincubated for 30 min and then transferred into fresh media every 10 min by means of a micromanipulator. Each preparation was studied over a total of eight to twelve 10 min incubation periods. In certain experiments the KCl concentration was increased, but the osmolarity was not concomitantly corrected since Douglas & Poisner (1964*a*) have shown that hypertonic media fail to increase hormone output from the isolated neurohypophysis. TTX (Sankyo, Tokyo) was added to the medium in other experiments.

Determination of hormones in the incubation media. After incubation, media were stored at 4° C for not more than a week and assayed, against synthetic oxytocin standards (Syntocinon^R Sandoz), by the rat milk-ejection method described by Bisset, Clark, Haldar, Harris, Lewis & Rocha e Silva (1967). Lactating rats were anaesthetized with urethane (125 mg/100 g body wt.) 5–10 days after parturition and an inguinal or abdominal mammary gland was cannulated with a fine polyethylene tube. Pressure changes were recorded through a Statham Db 23 pressure transducer. Standards (diluted in Locke solution) and unknowns were injected (volumes 0·05–0·3 ml.) through a cannulated jugular vein. The threshold dose was 100–200 μ-u. oxytocin. Synthetic lysine-vasopressin (Sandoz) was approximately 4 times less potent than oxytocin (compared unit per unit); no attempt was made to differentiate between the two octapeptides, and all results are expressed in fractions of international units of oxytocin. TTX (10^{-7} g/ml.) did not modify the milk ejection response of the assay animal to oxytocin standards.

Electrical stimulation. During stimulation periods (1 min, unless otherwise stated) the preparation was raised so that the inferior pole of the neural lobe remained in contact with the incubation medium by surface tension. Electrical stimuli were applied between the platinum wire carrying the preparation and a platinum wire coil dipping into the incubation medium. Trains of rectangular biphasic pulses (6 msec duration, 0·5–1·0 mA intensity; cf. Harris, Manabe & Ruf, 1969) were delivered at various frequencies by means of a 'constant current' stimulator (Nuclear Chicago, model no. 7153). In order to obtain stable conditions the supply of gas was interrupted during stimulation but restored during the remainder of the incubation period (9 min).

Recording of a compound action potential from the neural lobe. Ten additional experiments were done on a preparation composed of the entire hypophysis and the pituitary stalk; potential changes were recorded from the neurohypophysis in response to electrical stimulation of the pituitary stalk. With the neural lobe facing upwards, the anterior pituitary was pinned to the bottom of a small lucite chamber constantly perfused with Locke solution (37° C). Bipolar silver electrodes (60 μ diameter, insulated with Teflon except at the tips) were used for stimulation and for recording. The stimulating electrodes were placed across the pituitary stalk, the recording electrodes on the most distal part of the neural lobe, 2–3 mm away from the site of stimulation. Potentials were recorded via an a.c. amplifier (Tektronix 2A 61). In order to minimize the stimulation artifact, short (0·05–0·1 msec) monophasic rectangular pulses were used.

RESULTS

1. *Effects of electrical stimulation of the pituitary stalk on hormone release.* Following a 30 min preincubation period, resting hormone release during consecutive 10 min incubation periods was consistently low ($\leqslant$ 1·5 m-u./lobe.10 min) over at least a 2 hr period. The viability of the preparation *in vitro* was ascertained by its response to electrical stimulation at various times. A typical experiment is illustrated in Fig. 1, which shows that repeated exposure of the neurohypophysis to the same type of electrical

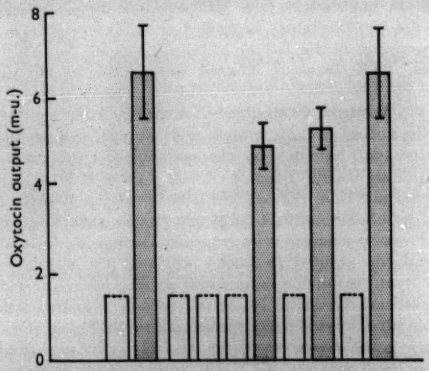

Fig. 1. Basal hormone release from single neural lobes following a 30 min preincubation period and effect of electrical stimulation of the pituitary stalk. Open columns: resting output during successive 10 min incubation periods in the absence of electrical stimulation; values are estimates of upper limits, the output being too small to be assayed with accuracy. Hatched columns: output following electrical stimulation for 1 min at 1·0 mA, 50 c/s. Mean $\pm$ S.E. of four experiments.

stimulation always led to increased hormone release. However, some variation of the hormone content of incubation media was found; this may be caused by changes in responsiveness of the neural lobe to repeated stimulation and/or the limited precision of the bio-assay used.

2. *Comparison of the effects of given numbers of stimuli applied at various frequencies.* In the above experiments the electrical stimulus consisted of 1 min trains delivered at 50 c/s, i.e. containing 3000 stimuli. It became of interest to see whether hormone release depended exclusively on the total number of stimuli delivered to the pituitary stalk or whether the frequency of stimulation *per se* was also a determinant of the release process. To this end, the pituitary stalk was stimulated with trains of 500, 1000, 1500 and 2000 stimuli delivered at different frequencies so that the train duration

was either 10, 30, 60 or 180 sec. As before, the incubation periods were 10 min each and eight incubations were carried out on each of ten separate lobes. Each of the different stimuli was tested on five separate occasions, their order of presentation being determined by a random design. The incubation samples were identified by code until bioassays were completed.

The results from these eighty incubation periods are shown in Fig. 2. Detectable amounts of hormones were liberated at all frequencies tested (2·8–200 c/s), but certain combinations of parameters were clearly more

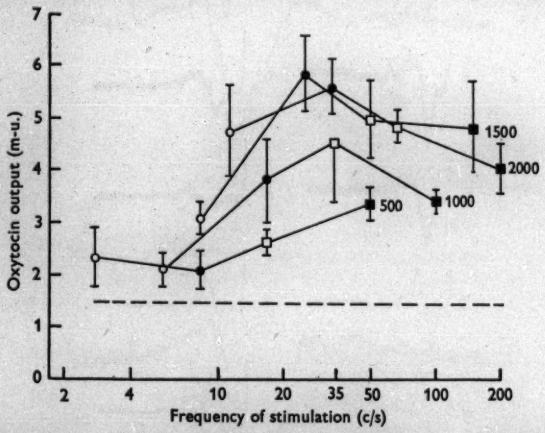

Fig. 2. Influence of total number of stimuli and frequency of stimulation on hormone release. Trains of 500, 1000, 1500 and 2000 stimuli (1 mA, 3 msec) were delivered at various frequencies resulting from train durations of 3 min (○), 1 min (●), 30 sec (□) and 10 sec (■). Each point represents mean ± S.E. resulting from five separate stimulations. Dashed line: estimated resting release. Note tendency to increased hormone output with higher numbers of stimuli. In the lower frequency range (< 35 c/s) given numbers of stimuli may liberate more hormone if the frequency of stimulation is increased.

effective than others, provided that the frequency of stimulation did not exceed *ca.* 35 c/s. Thus 1000 stimuli became significantly ($P < 0{\cdot}05$, Student's t test) more effective when the frequency of delivery was increased from 5·6 c/s (3 min train) to 33·3 c/s (30 sec train). Likewise, 1500 stimuli liberated significantly ($P < 0{\cdot}001$) more hormone when the frequency passed from 8·3 c/s (3 min train) to 25 c/s (1 min train). A somewhat similar trend was observed for 500 stimuli and for 2000 stimuli, but none of these increments were statistically significant ($P > 0{\cdot}1$). Differences between a lower and a higher frequency range were also found with respect to the influence of the total number of stimuli on hormone release;

Fig. 2 shows that in the lower frequency range (< 35 c/s) release was approximately proportional to the number of stimuli, but that in the higher frequency range additional stimuli became less effective. Although the figure reveals a fairly consistent trend, the low accuracy of results obtained by this approach should be stressed.

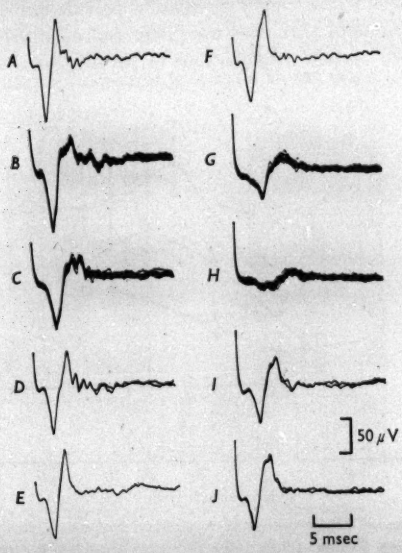

Fig. 3. Influence of frequency of stimulation on the shape of the compound action potential recorded from one neurohypophysis *in vitro*. The stalk was stimulated with pulses of 0·05 msec, 4·0 mA. Only the end of the stimulation artifact appears on the traces. The responses were recorded in the order *A*–*J*. *A* and *F*: controls (0·1 c/s). *B* and *C*: approximately 10 sec and 30 sec after beginning of stimulation with 12 c/s. Low-frequency stimulation (0·1 c/s) was resumed immediately after *C*. *D* and *E*: 3 min and 10 min after *C*. *G*–*J*: similar sequence, except stimulation with 50 c/s in *G* and *H*. Note decreased amplitude of the response to stimulation with 50 c/s.

3. *Characteristics of compound action potentials evoked by electrical stimulation of the pituitary stalk.* In view of the above results, the question arises whether the hormone release evoked by electrical stimulation of the stalk is mediated by propagated action potentials and, if so, how these are correlated with the electrical stimulus on one hand and with release on the other. A compound action potential is easily recorded from the neurohypophysis following electrical stimulation of the stalk (Figs. 3, 4).

Typically, the latency period is of the order of 5 msec, which is compatible with the slow velocity (< 1 m/sec) conduction described for this system (Yagi, Azuma & Matsuda, 1966; Ishikawa, Koizumi & Brooks, 1966; Dyball & Koizumi, 1969; Sundsten, Novin & Cross, 1970; Ishida, 1970). In normal Locke solution the amplitude of the potential remains stable for several hours if the stalk is stimulated at low frequencies (< 1 c/s), but diminishes within seconds during stimulation with higher frequencies

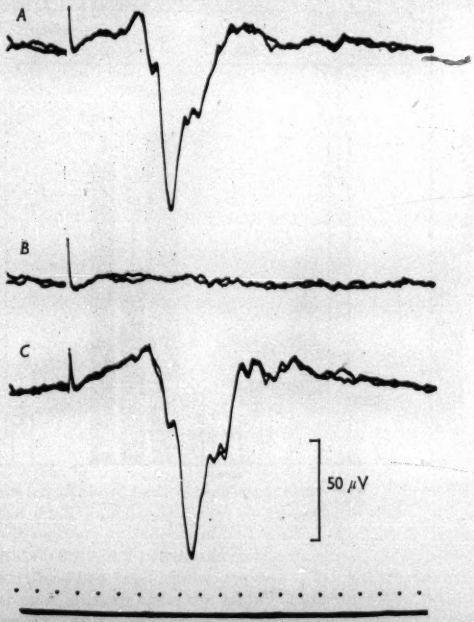

Fig. 4. Effect of TTX on the compound action potential. Each record represents two superimposed oscilloscope traces obtained during stimulation of the pituitary stalk (0·05 msec, 2·0 mA). *A*, in normal Locke solution; *B*, 5 min after addition of TTX (10^{-7} g/ml.) to the incubation medium; *C*, 100 min after re-exposure to normal Locke solution. Horizontal calibration: msec.

(five expts.). Fig. 3 shows this reduction of amplitude, which was moderate when the fibres were stimulated with 12 c/s, but marked when stimuli of 50 c/s were employed. Stimulation with 50 c/s reduced the amplitude of the compound action potential to one-seventh of the control value by the end of a 30 sec stimulation period. About 80 % recovery was observed 10 min after the cessation of the stimulus. The compound action potential

was found to disappear in 'sodium-free' Locke solution (two expts.) as well as after the addition of TTX ('TTX-Locke solution', three expts.) to the incubation medium (Fig. 4*B*). The blocking effect of TTX was reversible (Fig. 4*C*).

4. *TTX and hormone release.* In normal Locke solution electrical stimulation of the pituitary stalk increases the hormone output approximately

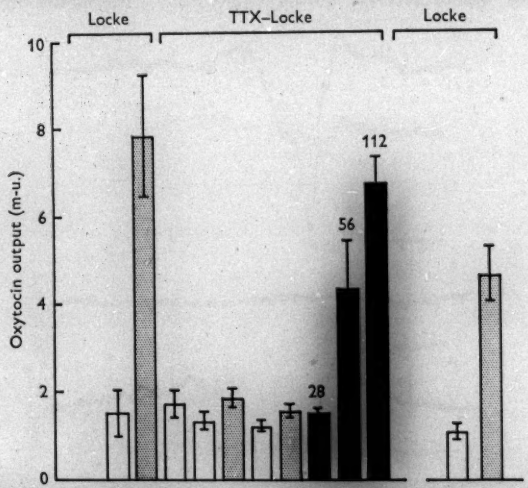

Fig. 5. Effect of TTX on hormone release evoked by electrical stimulation of the pituitary stalk or exposure to excess potassium. Each column represents output during consecutive 10 min incubation periods; open columns, resting state; hatched columns, stimulation at 1·0 mA, 50 c/s, during 1 min; filled columns, presence of excess potassium; figures indicate concentration of KCl (mM) in the medium. Values are mean ± S.E. of four experiments. Note failure of electrical stimulation to increase output after addition of TTX (10^{-7} g/ml.), while increased potassium promotes increased release. The last two incubations (normal Locke solution) were carried out after a 50 min interval and show partial recovery of response to electrical stimulation.

fivefold (Fig. 5). This increase was not found 20 or 40 min after the lobe had been transferred to 'TTX-Locke solution', although the resting release continued as in controls. This indicates that resting release is independent of action potentials and suggests that hormone release, whether resting or evoked, may be totally unrelated to the increase in sodium conductance responsible for the generation of the action potential. Evidence that the release mechanism remains operational in 'TTX-Locke

solution' derives from the response of neural lobes exposed to excess potassium (Fig. 5). Exposure to 56 mM-KCl doubled hormone output of TTX-poisoned neural lobes, and a further increase in output was seen with 112 mM. In two cases in which external calcium was omitted from the 'TTX-Locke solution' excess potassium was without effect.

Our finding that TTX does not prevent the stimulation of release by excess potassium (provided calcium is present in the incubation medium) led us to reinvestigate Ishida's (1967) claim that in 'TTX-Locke solution'

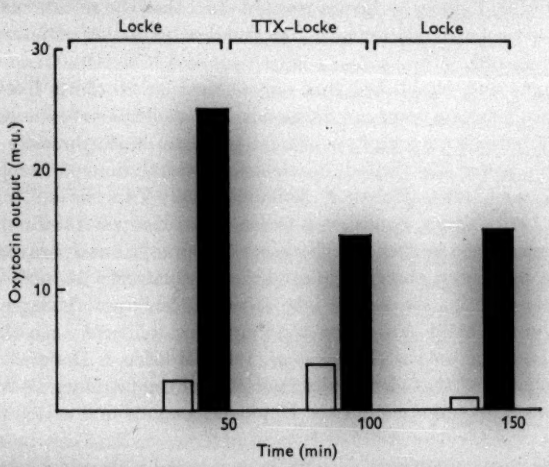

Fig. 6. Effect of tetrodotoxin (10^{-7} g/ml.) on hormone release evoked by excess potassium. Columns represent output/five neural lobes. 10 min (cf. Ishida, 1967, p. 310). Open columns, controls (5·6 mM-KCl); filled columns, 56 mM.

an 80 % suppression of the vasopressin release evoked by excess potassium (56 mM) is observed. In one experiment we have reproduced her experimental conditions exactly, except that oxytocin rather than vasopressin was determined. Fig. 6 shows that when TTX was added to Locke solution containing 56 mM-KCl the output of the hormone was only 60 % of that from control preparations in the same potassium concentration. The response to high potassium was not restored when the lobe was transferred to 'TTX-free Locke solution' with high KCl.

DISCUSSION

In this study an attempt has been made (i) to quantify the relationship between electrical stimulus and release of hormone from the isolated neurohypophysis, and (ii) to dissociate the action potential generating mechanisms from the release process. It was found that both total number of stimuli and their frequency are important determinants of hormone release, provided that the frequency of stimulation does not exceed a critical value. Further, evidence was obtained that the release mechanism at the nerve terminals remains functional after action potentials have been eliminated.

It is now well established that neuroendocrine neurones, like conventional ones, generate propagated action potentials. The cell bodies giving rise to the hypothalamo-neurohypophysial tract are antidromically invaded by action potentials elicited by electrical stimulation of the pituitary stalk (Kandel, 1964; Dyball & Koizumi, 1969; Yamashita, Koizumi & Brooks, 1970; Nővin, Sundsten & Cross, 1970; Kelly & Dreifuss, 1970*a*), and changes in the frequency of discharge of supraoptic and paraventricular neurones have been observed in conditions associated with increased hormone release from the neural lobe (Brooks, Ishikawa, Koizumi & Lu, 1966; Dyball, 1970). From the blocking effect of local anaesthetics on evoked hormone output (Haller *et al.* 1965; Mikiten & Douglas, 1965) it has been inferred that electrical stimulation of the pituitary stalk *in vitro* may also set up action potentials. Moreover, a compound action potential which is dependent on the presence of external sodium can be recorded from the neurohypophysis *in vitro* in response to electrical stimulation (Yagi *et al.* 1966; Ishida, 1970). Our finding that both this compound action potential and the evoked release of hormones from the neural lobe are abolished by TTX gives strong support to the view that action potentials initiate the release process *in vitro*, since TTX interferes selectively with the increase in sodium conductance responsible for the generation of action potentials (Narahashi, Moore & Scott, 1964).

Several authors have studied the effect of electrical stimulation on the release of neurohypophysial hormones in recent years, but few quantitative observations on the influence of stimulation parameters have apparently been made. Harris *et al.* (1969) have shown that the milk-ejection response in the lactating rabbit caused by electrical stimulation of the supraoptico-neurohypophysial tract is critically dependent on the frequency of stimulation and is not obtained at frequencies below 25–30 c/s. A similar threshold has been found in the lactating rat (Dreifuss & Ruf, 1971). According to recent studies by Ishida (1970) the most effective frequency of stimulation for the release of hormones from the *isolated* neurohypophysis is 10–20 c/s;

hormone output is said to decrease markedly above and below this optimal frequency. The above experiments may be misleading in the sense that changes in frequency are inevitably accompanied by changes in the total number of stimuli delivered during a given stimulation period. In this study, we have tried to control the latter variable by applying predetermined numbers of stimuli at various frequencies. Our results indicate that the impulse-carrying capacity of the hypothalamo-neurohypophysial tract is an important determinant of hormone release under experimental conditions. Stimuli delivered at frequencies of $\leqslant$ 35 c/s appear to be easily transmitted to the terminals of the C-fibre tract and to release hormones as a function of *both* their total number and their frequency of application (Fig. 2). Stimuli of higher frequency (e.g. 50 c/s) are only transmitted during a short time interval ($> 10 < 30$ sec), and their efficiency with respect to hormone release declines during and after this period. Since the small (0·2–0·5 μ in diameter) unmyelinated fibres of the pituitary stalk have a high surface/volume ratio, repeated propagation of action potentials may increase the internal sodium concentration while diminishing the internal potassium concentration at the same time (cf. Keynes & Ritchie, 1965) and thus lead to partial or total inactivation of the impulse generating mechanism (see Fig. 3). Observations made *in vivo* are in keeping with these considerations (Novin *et al.* 1970; Kelly & Dreifuss, 1970*b*).

An interesting phenomenon is the influence of frequency of stimulation in the lower (< 35 c/s) frequency range, where impulse transmission appears assured. Here, identical numbers of stimuli are more efficient in promoting hormone release if they are applied at higher frequencies (Fig. 2). The finding is reminiscent of the process of 'facilitation' observed at certain chemical synapses, where the release of neurotransmitters is similarly dependent on the interstimulus interval (for references, see Hubbard, 1970). At these synapses, facilitation of transmitter release has been attributed to a residual fraction of calcium remaining at an active intracellular site when further impulses reach the nerve terminals (cf. Katz & Miledi, 1968).

Yet another parallel between the release mechanisms for neurohypophysial hormones on one hand and neurotransmitters on the other has been established by the use of TTX. At synaptic terminals, TTX abolishes action potentials but affects neither the spontaneous, quantal release of neurotransmitter nor the increased liberation in response to depolarization (Katz & Miledi, 1967*a*, *b*; Hubbard, 1970). We have shown that in the presence of TTX the resting release of neurohypophysial hormones is similarly unaffected and that graded depolarization by excess potassium still induces a proportional enhancement of hormone output. We feel that

for the understanding of the release process the maintenance of a proportional response to external potassium is more significant than the overall reduction of its size (Ishida, 1967; cf. Fig. 6), since the latter might be explained by the absence of conducted action potentials and/or the exhaustion of a readily accessible hormone pool (Thorn, 1970). The experimental uncoupling of the release process from the action-potential generating mechanism by the use of TTX should prove useful for further studies of neurohypophysial secretion.

This work was supported by grant no. 5340.3 from the Swiss National Foundation for Scientific Research and grant no. 117 from the Hoffmann–La Roche Foundation. The technical assistance of Miss R. E. Bianchi is acknowledged. J. S. K. was supported by IBRO/Unesco and the Medical Research Council of Canada.

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J. Physiol. (1971), **215**, *pp.* 819–848
With 17 *text-figures*
Printed in Great Britain

THE MECHANISM OF ACETYLCHOLINE RELEASE FROM PARASYMPATHETIC NERVES

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(*Received 13 January 1971*)

SUMMARY

1. The output of acetylcholine from the plexus of the guinea-pig ileum longitudinal strip has been used to study the mechanism of acetylcholine release. From the effects of hexamethonium and tetrodotoxin, it was inferred that 60 % of the normal resting output is due to propagated activity in the plexus, and 40 % to spontaneous release. Tetrodotoxin virtually abolishes the increase in output in response to electrical stimulation.

2. Resting acetylcholine output is increased when the bathing medium is changed in the following ways:

(*a*) sodium replacement by sucrose, trometamol or lithium;

(*b*) addition of ouabain or *p*-hydroxymercuribenzoate (PHMB), or withdrawal of potassium;

(*c*) the combination of PHMB and partial sodium replacement;

(*d*) addition of potassium; this increase in output becomes greater in the absence of sodium.

3. The resting output is virtually abolished by calcium withdrawal, and is restored by barium substitution for calcium. It is also reduced by raising the magnesium concentration.

4. The enhanced resting output in response to sodium withdrawal also occurs in the absence of calcium.

5. Cooling to 5° C greatly reduces both the resting output and the output in response to raised potassium concentration or to electrical stimulation.

6. The increase in resting output due to potassium excess is slight up to 25 mM $[K^+]_o$, but increases thereafter with about the fourth power of the potassium concentration; it is resistant to tetrodotoxin.

7. Synthesis of acetylcholine by the longitudinal strip is increased when output is enhanced by electrical stimulation, by potassium excess or by

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addition of barium, so that the acetylcholine content of the strip is maintained approximately normal. Synthesis is reduced, in relation to output, by potassium lack or by treatment with ouabain, and is virtually abolished by sodium withdrawal.

8. The theory is discussed that acetylcholine release depends on inhibition of the activity of a ($Na^+ + K^+ + Mg^{2+}$)-activated ATPase at the axonal membrane.

INTRODUCTION

The principal studies of the mechanism of acetylcholine release have been made on the neuromuscular junction and on the autonomic ganglion. To the classical evidence that established the theory of chemical transmission has been added new evidence made possible by the discovery of quantal release of acetylcholine at the motor end-plate and the development of new electrophysiological methods (*v.* Katz, 1962). But there is less evidence for parasympathetic structures. Acetylcholine release in the salivary gland was studied by Emmelin & Muren (1950); and there is a considerable amount of work on acetylcholine output from the alimentary tract, but this has been complicated by uncertainty as to the origin of the acetylcholine.

The finding that, in the longitudinal strip of guinea-pig ileum, acetylcholine content and release are restricted to Auerbach's plexus, and that the smooth muscle neither contained nor released acetylcholine (Paton & Zar, 1968) made possible an examination of the factors controlling acetylcholine release by this parasympathetic plexus. It has already been used for a study of the action of catecholamines (Paton & Vizi, 1969). The main advantage of the preparation is that it is suitable, from the thinness of the strip, for *in vitro* study, and that relatively large amounts of acetylcholine are released which can be directly assayed.

METHODS

The methods described by Paton & Zar (1968) and Paton & Vizi (1969) were used for the preparation and mounting of longitudinal muscle strips of guinea-pig ileum, and for assay of acetylcholine output and tissue content. The weight of the strips varied from 18 to 80 mg, depending on the animal and on the method of preparation. The composition of the normal Krebs solution and its modification were as follows. (*a*) Normal Krebs (mM), NaCl 113; KCl 4·7; $CaCl_2$ 2·5; KH_2PO_4 1·2; $MgSO_4$ 1·2; $NaHCO_3$ 25; and glucose 11·5. (*b*) Sodium-substituted solutions contained equimolar amounts of sucrose, lithium chloride, or trometamol (Tris) chloride; the $NaHCO_3$ was replaced with $KHCO_3$, KCl and KH_2PO_4 being omitted. (*c*) Potassium-free solutions were prepared by omitting KCl and KH_2PO_4, either without adjusting osmolarity (as in the experiment of Fig. 6) or by appropriate NaCl reduction as in the experiments of Figs. 11 and 12. (*d*) Potassium-rich solutions were made by adding KCl, up to 49 mM. (*e*) Calcium-free solutions were made by omitting $CaCl_2$. (*f*) For

barium-enriched solutions, $BaCl_2$ was added to calcium-free Krebs, to a final concentration of 1·25 mM, and magnesium chloride replaced an equimolar amount of magnesium sulphate. Before assay of such solutions for acetylcholine content, barium was precipitated by adding Na_2SO_4 to a concentration of 15 mM of Na_2SO_4 in the assay fluid; after shaking, the fluid was filtered and assayed directly. In all assays, the standard acetylcholine solutions were made up in appropriately modified Krebs solution.

In experiments at low temperature, the organ bath was arranged in a trough of ice; the temperature was reduced to 3–5° C.

Drugs. The drugs used were acetylcholine chloride or bromide (values given in the paper are cited as acetylcholine salt, mol. wt. 181·7), physostigmine sulphate (Burroughs Wellcome & Co.), (±)-adrenaline bitartrate (Burroughs Wellcome & Co.), (−)-noradrenaline bitartrate (Koch Light Laboratories Ltd.), hexamethonium bromide, morphine sulphate, cocaine hydrochloride (May & Baker Ltd.), procaine hydrochloride, sodium *p*-hydroxymercuribenzoate (Sigma Chemical Co.), tetrodotoxin (Sankyo), ouabain (Strophanthidin, B.D.H.), and trometamol (Tris; tris-(hydroxymethyl)-aminomethane, Sigma Chemical Co.) which was converted to the chloride by titration with hydrochloric acid at pH 7·3.

RESULTS

Analysis of the resting release of acetylcholine

For the analysis of release mechanisms, there is a choice between the use of resting or of evoked output. With the longitudinal strip, the resting output, while varying from one preparation to another, is reasonably constant for a given preparation for long periods (see, for example, Fig. 9). The mean value for eighty-four experiments was $51 \pm 1{\cdot}5$ ng/g.min. The evoked output, however, usually changes as stimulation is prolonged, in a manner varying with the stimulation used. Further, if changes in the ionic environment are made, difficulty arises with field stimulation in securing the same conditions of nervous excitation as are used in control periods. Accordingly, even though resting output is lower in magnitude than that obtainable by excitation, it has been used for this analysis.

The first question that arises is how far what is termed resting output originates from propagated activity in cholinergic nerves, and how far it corresponds to what will be termed 'spontaneous' release from nerve fibres free of action potentials. It is useful in considering this problem to distinguish three possible types of cholinergic fibre; 'extrinsic' fibres, i.e. those with cells of origin outside the strip; 'interneuronal' fibres, i.e. those with cell bodies located within the plexus and synapsing with other ganglion cells; and 'pre-effector' fibres, i.e. those with cell bodies located in the plexus, whose nerve endings come into relation not with nerve cells but smooth muscle. The terms pre- and post-ganglionic are confusing in this context since both terms could be applicable to an interneuronal axon. Interneuronal cholinergic cell bodies could be uninnervated (activated

perhaps by axon reflex), or innervated by non-cholinergic fibres (e.g. of local sensory origin), or innervated by cholinergic fibres (possibly as part of a re-entrant cholinergic network). With each of the types of cholinergic fibre, acetylcholine release could occur either spontaneously or due to action potentials, so that six possible fractions of acetylcholine output can be distinguished (Fig. 1).

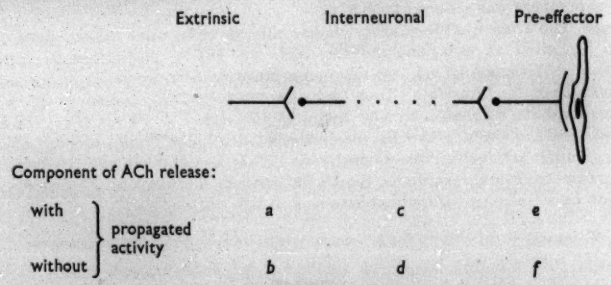

Fig. 1. Diagram of possible sources of acetylcholine (ACh) in the nerve plexus of the longitudinal muscle strip. See text.

TABLE 1. Inhibition by drugs of resting acetylcholine release from longitudinal strip: % ± S.E. Number of experiments in brackets. Exposure time, 20 min

	g/ml.	
Hexamethonium	5×10^{-5}	61·9 ± 4·8 (4)
Hexamethonium	10^{-4}	53·0 ± 6·6 (5)
Tetrodotoxin	5×10^{-7}	54·8 ± 3·2 (6)
Hexamethonium + Tetrodotoxin	10^{-4} + 5×10^{-7}	61·7 ± 2·4 (3)
Adrenaline*	10^{-6}	71·2 ± 5·7 (6)
Procaine	2×10^{-5}	83·3 (2)
Morphine	10^{-6}	81·5 ± 2·7 (4)
Hexamethonium + Adrenaline	10^{-4} + 5×10^{-7}	80·2 ± 10·1 (5)
Hexamethonium + Morphine	10^{-4} + 10^{-6}	81·7 ± 6·2 (8)

* Taken from Paton & Vizi (1969).

Table 1 shows the action of a number of drugs on resting output. First, hexamethonium in a concentration of 5×10^{-5} or 10^{-4} g/ml. reduced the resting output by about 57 %. The concentration used is 5–10 times greater than that required to abolish both the peristaltic reflex (Paton & Zaimis, 1949) and the spontaneous activity of the guinea-pig ileum (Feldberg, 1951), and must be supposed to abolish cholinergic ganglionic transmission

in the strip. On this basis, the residual output after hexamethonium, about 40% of normal, must at most represent (using the symbols of Fig. 1) fractions *a*, *b*, *d* and *f*.

Secondly, tetrodotoxin, 5×10^{-7} g/ml. (1·6 μM), reduced resting output in six experiments by 55%. Fig. 2 shows further experiments on the dose–response curve of the inhibition; it was readily detectable at 0·15 μM and is virtually maximal at concentrations of 1 μM or above. From what is now known of the properties of tetrodotoxin (Narahashi, Moore & Scott, 1964;

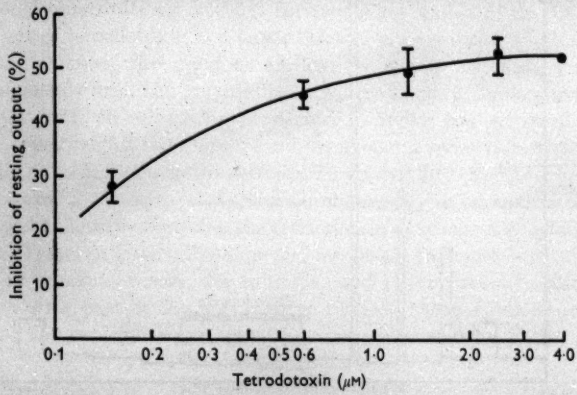

Fig. 2. Inhibition of resting acetylcholine output from the longitudinal muscle strip of guinea-pig ileum by tetrodotoxin. Abscissa: concentration of tetrodotoxin (μM). Ordinate: % inhibition of the resting acetylcholine output. Mean of six experiments; vertical bars indicate S.E. The control resting output was $44·7 \pm 3·2$ mg/g.min.

Katz & Miledi, 1967) it must be assumed that all propagated activity in nerve fibres is abolished, but that spontaneous quantal release of acetylcholine at nerve terminations is retained. Fig. 3 shows that tetrodotoxin abolishes the response to stimulation, at rates both of 0·3 Hz and 10 Hz, as well as reducing resting output. If it is supposed that quantal activity such as occurs at the end-plate is responsible for all spontaneous release unaccompanied by action potentials (and therefore is all tetrodotoxin-resistant), then the residual output under resting conditions after tetrodotoxin, about 45% of normal, must represent the fractions *b*, *d* and *f*. The quantitative similarity of the effect of hexamethonium to that of tetrodotoxin is interesting, and indicates that acetylcholine release of extrinsic origin (i.e. fraction *a*) is negligible. This is further supported by three experiments in which the output in the presence both of hexamethonium (10^{-4} g/ml.) and of tetrodotoxin (5×10^{-7} g/ml.) was determined; output

was reduced by 61·7%, a value not differing significantly from that obtained with either drug alone. The insignificance of an extrinsic component is in any case probable, in the light of what is known of the distribution of the vagus to the alimentary tract in the cat (Agostini, Chinnock, de Burgh Daly & Murray, 1957). The results also suggest that propagated activity

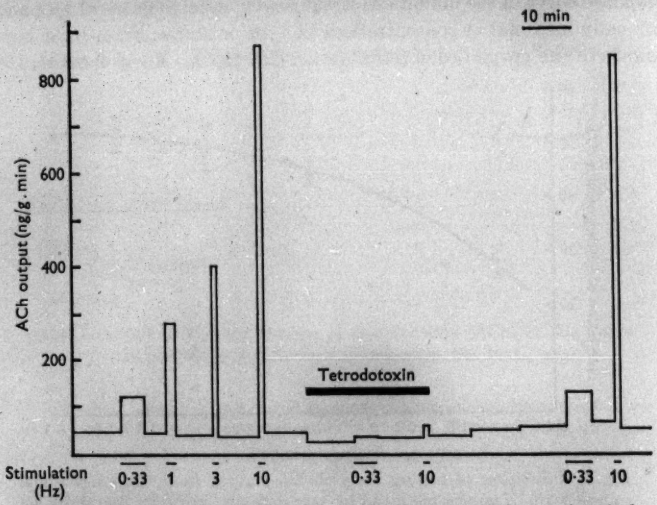

Fig. 3. Inhibition by tetrodotoxin of acetylcholine output due to electrical stimulation. Longitudinal muscle strip of guinea-pig ileum in eserinized Krebs solution. Tetrodotoxin (5×10^{-7} g/ml.) present as indicated. The values of acetylcholine output (ng/g.min) are means of two experiments. The preparation was stimulated for periods of 5 min at 0·33 Hz, 2 min at 1 Hz and 1 min at 10 Hz. Note the almost complete but reversible inhibition of acetylcholine output by tetrodotoxin.

resulting in acetylcholine release in the resting strip arises only from activity by cholinergic mechanisms; if this were not the case, then cholinergic neurones activated by some other mechanism would remain active in the presence of hexamethonium, but not of tetrodotoxin, and the latter would have a greater effect in reducing acetylcholine output. We have assumed, therefore, that 40% of the resting output is due to spontaneous activity and the remainder arises from propagated activity evoked by local cholinergic mechanisms.

The action of catecholamines, cocaine, procaine and morphine

Certain drugs are able to depress resting output still further. In a previous paper (Paton & Vizi, 1969) it was reported that adrenaline, 10^{-6} g/ml.,

reduced resting output by $71{\cdot}2 \pm 5{\cdot}7\%$. Procaine and morphine produce similar inhibitions: procaine 2×10^{-5} g/ml., 83·3% (two experiments); morphine 10^{-6} g/ml., $81{\cdot}5 \pm 2{\cdot}7$ (four experiments). In higher concentrations, morphine does not exert any greater effect. Similar inhibitions have been found with whole intestine; thus Johnson (1963) has found that procaine and cocaine reduced the output from whole ileum by about 60–85%, and we have confirmed that cocaine ($1{\cdot}5 \times 10^{-4}$ g/ml.) reduces output from whole ileum by $66{\cdot}7 \pm 4{\cdot}6\%$. But these data on whole gut cannot be considered quantitatively without corresponding studies of the effects on whole gut of tetrodotoxin and hexamethonium.

In the light of the previous section, the ability of these drugs to depress output from the longitudinal strip by 70–80% implies that they must act both on release by propagated activity and on spontaneous release. They might, therefore, be annulling spontaneous release and only partially reducing propagated activity. This possibility can be excluded by the fact that if the strip is exposed simultaneously to adrenaline or morphine and to hexamethonium, the reduction in output is not significantly greater (Table 1). With adrenaline and morphine, therefore, and probably also with procaine, release due to propagated activity must be abolished, together with part of the spontaneous release. The possible mechanism involved for catecholamine action has been discussed by Paton & Vizi (1969).

The mechanism of release

Effect of sodium replacement. Evidence has been put forward (Birks, 1963) that the release of acetycholine in autonomic ganglia and at the neuromuscular junction depends on a rise of internal sodium concentration in the nerve ending, and this has led to a later suggestion (Birks & Cohen, 1968) that it acts not directly, but by increasing calcium influx. We have tested such a role of sodium on output from the longitudinal strip by exposing the strip to sodium-deficient media. Fig. 4 shows the change in output which follows treatment with media in which the sodium was wholly replaced with trometamol (Tris), with sucrose, or with lithium. In each case, a considerable rise in output developed, most rapid and largest with lithium substitution. This was an unexpected result, both in the light of Birks's findings, and because it would be expected that a fall in output would in any case result from an abolition of propagated nervous activity following sodium withdrawal. This latter effect can in fact be seen with reduced sodium concentrations. Fig. 5 shows the output with media containing no sodium, 4% normal sodium, and 20% normal sodium, with sucrose substitution. With 20% sodium, a pure depression of output to about 20–30% of normal occurs; with 4% an initial depression passes into an increased output; with zero sodium, the enhanced output already noted

was observed. But if lithium was used in place of sodium with the 20% medium, only an increase in output was observed. Under these last conditions, it must be assumed that propagated activity will be retained, since lithium is known to substitute in this respect fully for sodium. It follows that under conditions where propagated activity can persist, and sodium cannot enter the nerve fibre, acetylcholine output is enhanced. Any simple

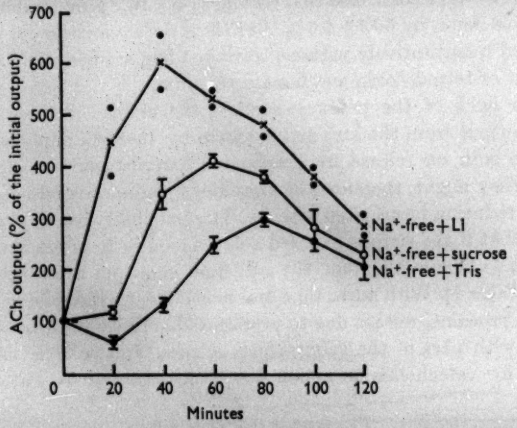

Fig. 4. Increase of resting acetylcholine output by total sodium deprivation. Eserinized longitudinal muscle strip of the guinea-pig ileum in modified Krebs solution. Sodium was replaced as indicated by lithium or sucrose or Tris (trometamol). For details see Methods. The initial rate of acetylcholine output in the presence of normal amount of sodium in Krebs solution has been taken to be 100 and the subsequent rates of acetylcholine release, on treatment with sodium-free solutions, have been expressed as % of the initial rate. Each point represents the resting acetylcholine output during the preceding 20 min. In two lower curves, bars give S.E. of mean of three experiments; upper curve drawn through means of two experiments, the individual values being shown.

correlation between output and rise in internal sodium concentration cannot therefore be accepted; and in fact the experiments quoted would support a view that a fall in axonal sodium concentration favoured release.

Another observation should be mentioned here. Fig. 6 showed the effect of sodium deprivation on the increase in output produced by raised external potassium concentration. This treatment enhances the acetylcholine release by up to twofold.

Effect of interfering with sodium extrusion. The converse experiments, with procedures calculated to raise intra-axonal sodium, by interfering with the sodium pump, were made with ouabain, reduced external potas-

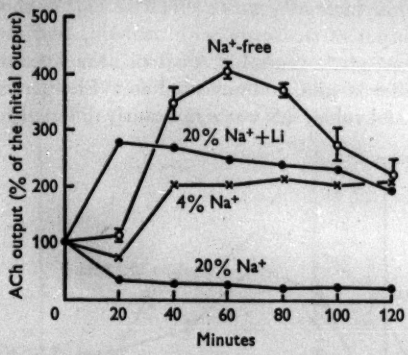

Fig. 5. Resting acetylcholine output from eserinized longitudinal muscle strip exposed to various concentrations of sodium in modified Krebs solution. Sucrose substitution except when lithium was used. Initial rate of acetylcholine output in presence of normal Krebs solution taken as 100 %. ○—○ sodium-free solution, three experiments; bars give S.E. ×—× 4 % normal sodium, one experiment. ●—● 20 % normal sodium, one experiment, and 20 % normal sodium, lithium substitution, one experiment.

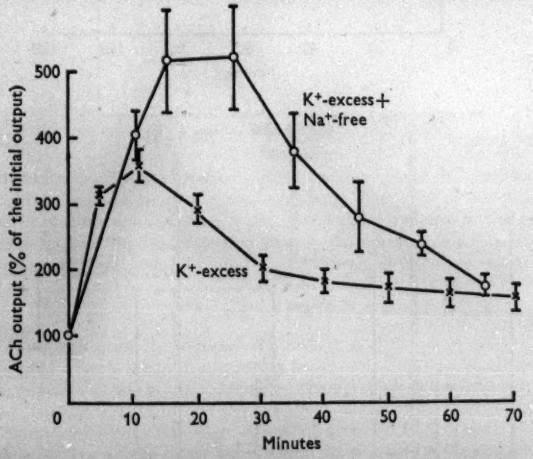

Fig. 6. Effect of complete sodium deprivation on acetylcholine output from eserinized longitudinal muscle strip of guinea-pig ileum exposed to excess potassium (40 mM). Tris was used as the substitute for the sodium. Control resting output, $103 \pm 7{\cdot}1$ ng/g.min, taken as 100 %. Bars give S.E. of mean of three experiments. Each point represents the acetylcholine output during the preceding period.

sium and *p*-hydroxymercuribenzoate (PHMB). Fig. 7*a* shows the effect on acetylcholine output of treatment with ouabain, 10^{-6} ($1{\cdot}4 \times 10^{-6}$ M) and 10^{-5} ($1{\cdot}4 \times 10^{-5}$ M) g/ml. With 10^{-6} ouabain, the maximum action was reached in the first 20 min, and increased acetylcholine output by $60{\cdot}6 \pm 10{\cdot}5$ % of the initial value: this was significantly different from the control,

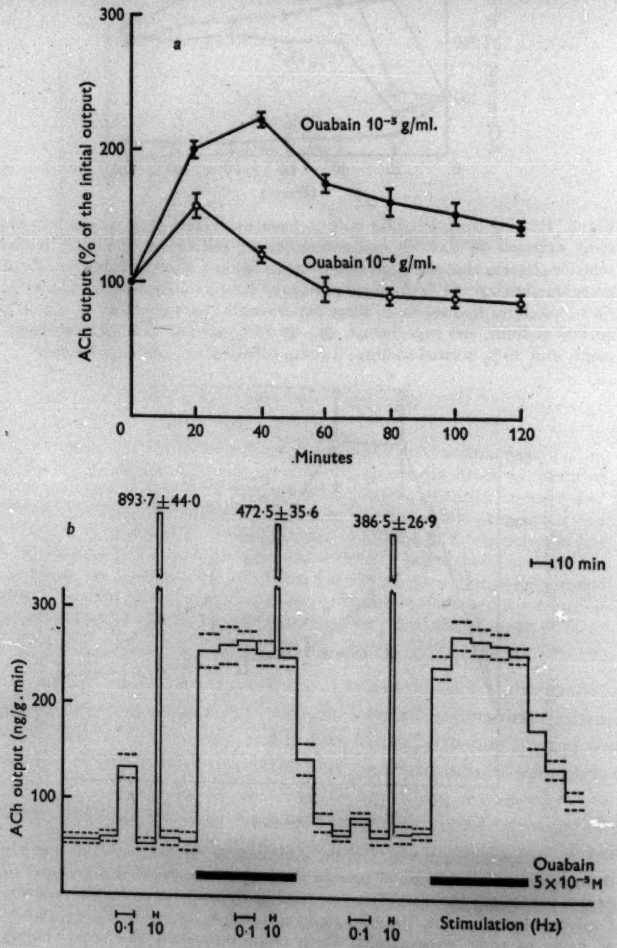

Fig. 7. For legend see opposite page.

but the increase in output in the following period, by 23 % ± 6·7, was not statistically significant.

With potassium-free solution (Fig. 8) the maximum effect was reached in the period from 90 to 120 min after removal of potassium, output being

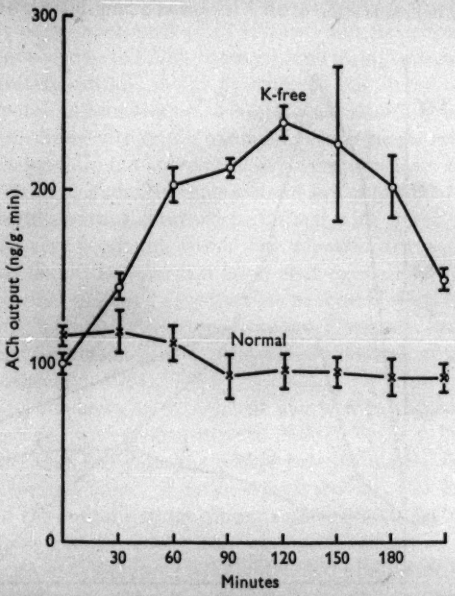

Fig. 8. Increase of acetylcholine output as a result of K_o^+ deprivation. Longitudinal muscle strip of guinea-pig ileum. The rate of acetylcholine output is plotted against time (min). Each point represents the acetylcholine output during the preceding 30 min. Potassium-free Krebs solution was prepared by omitting KCl and KH_2PO_4.

Legend to Fig. 7.

Fig. 7. Augmentation by ouabain of resting acetylcholine output from longitudinal muscle strip of guinea-pig ileum. (*a*) Time course of ouabain action. Abscissa: time in min. Ordinate: change of acetylcholine output in percent; the initial rate of acetylcholine output has been taken to be 100. Each point represents the mean value of acetylcholine output in three experiments during the preceding 20 min. Bars indicate the S.E. (*b*) The effect of ouabain (5×10^{-5} M) on the acetylcholine output under conditions of rest and of stimulation. The values of acetylcholine output (ng/g.min) are the means of four experiments with identical schedules. Dashed lines indicate the S.E. errors. Stimulation periods were 10 min at 0·1 Hz, 1 min at 10 Hz. Note the increase of acetylcholine output during rest and the decrease of stimulation output as a result of ouabain.

increased from an initial value of $103{\cdot}3 \pm 6{\cdot}3$ ng/g.min to $243{\cdot}3 \pm 10{\cdot}0$ ng/g.min. The Figure also shows the resting output under normal conditions; the output over a period of 4 hr ranged from 96·5 to 116·5 ng/g.min, a variation of less than $\pm 7\%$ from the mean value over the period.

PHMB in a concentration of 10^{-5} M caused a long-lasting and significant

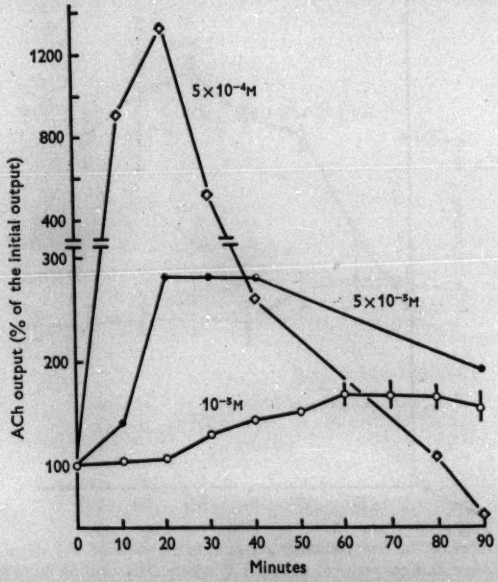

Fig. 9. Increase of acetylcholine output from the longitudinal muscle strip of guinea-pig ileum by sodium salt of *p*-hydroxymercuribenzoate (PHMB). The initial rate of acetylcholine output has been taken to be 100 and the subsequent rates of output, on treatment with PHMB, have been expressed as % of the initial rate. The following concentrations were used: ○ 10^{-5} M (three experiments, vertical bars indicate the S.E.), ● 5×10^{-5} M (one experiment). ◇ 5×10^{-4} (one experiment). Note that with 10^{-5} PHMB for the period from 20–30 min onwards the values differ significantly from the control ($P < 0{\cdot}01$). Each point represents the mean acetylcholine output during the preceding period.

increase in output, the increase reaching a maximum of $66{\cdot}6 \pm 9{\cdot}1\%$ in the 50–60 min period. The increase was statistically significant from the 20 to 30 min period onwards (Fig. 9). A larger increase, by 180% between 10 and 40 min, was produced in a single further experiment with 5×10^{-5} M-PHMB. In a final single experiment a concentration of 5×10^{-4} M caused an

increase in output of 810 % in the first 10 min, and a peak effect of 1260 %: this very greatly increased output could not be maintained, and at the end of 90 min output had fallen below normal.

A further set of experiments was made with a higher concentration of ouabain ($3{\cdot}7 \times 10^{-5}$ g/ml., 5×10^{-5} M) using identical dose and stimulation schedules for each experiment (Fig. 7*b*), in which its action on output to stimulation was also tested. This concentration of ouabain caused a fivefold increase in resting output: in the presence of this enhanced resting output, the response to stimulation at 0·1 Hz was undetectable and at 10 Hz reduced in size. After withdrawal of ouabain, responses to stimulation were present but still reduced. A subsequent exposure to ouabain again increased the resting output by about the same amount as previously. On withdrawing the drug, resting output returned to normal with a half-time of about 5–10 min, recovery becoming slower with repeated dosage.

It was also found, as described later, that calcium deprivation, in the presence of normal sodium and potassium, reduces output. This procedure, too, is likely to increase intra-axonal sodium (Frankenhaeuser & Hodgkin, 1957) and has been found to increase sodium content of smooth muscle (Paton & Rothschild, 1965).

Finally, Fig. 10 shows an experiment in which partial sodium deprivation was combined with treatment with PHMB. This was designed to test the possibility that acetylcholine release resulted merely from a disturbance (in either direction) of intra-axonal sodium. We have no direct knowledge of the actual sodium concentration; Krnjević (1955) estimated the intracellular sodium of cat sciatic nerve as 41 m-mole/kg water, and the interstitial sodium as 245 m-mole/kg water. Rang & Ritchie (1968) give the intra-axonal sodium content of rabbit desheathed vagus nerve as 76·6 m-mole/kg fibre water. For these experiments with non-myelinated nerve, if the sodium content of the external fluid were reduced to 20 % of the normal content, and simultaneously a sodium pump inhibitor applied, there should be a much smaller change in the sodium concentration of intraterminal axoplasm, through the great reduction in electrochemical gradient for sodium between the outside and inside of the nerves. Fig. 10 shows, however, that the addition of PHMB after low sodium treatment increased acetylcholine output to a peak value after 80 min of 3·5 times the initial level and 19 times the level attained in the low sodium medium.

These experiments show that an increase in acetylcholine output results from measures which would reduce intra-axonal sodium content (sodium withdrawal, with or without lithium substitution), or increase it (treatment with ouabain or PHMB, or potassium or calcium withdrawal), or tend to minimize such changes (the combination of partial sodium withdrawal with PHMB treatment). It seems, therefore, that some factor

other than the intra-axonal sodium content is involved in the control of acetylcholine release.

Effect of excess external potassium. A much simpler view, for which there is considerable support, is that acetylcholine output is a function of the membrane potential of the nerve endings. It has long been known that raised potassium concentrations increase acetylcholine output from the

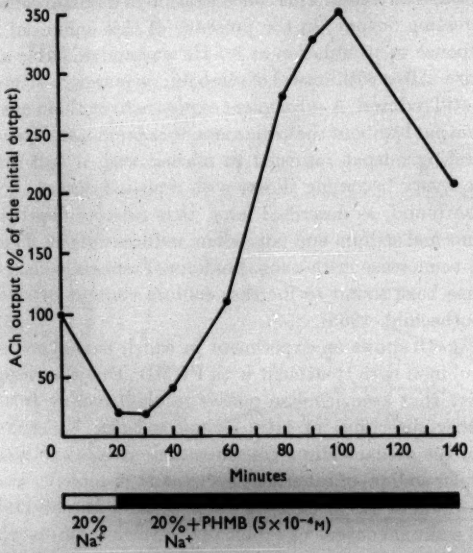

Fig. 10. Increases of acetylcholine output as a result of sodium salt of *p*-hydroxymercuribenzoate (PHMB) in a low sodium medium. 20 % sodium solution was prepared by replacing the rest of the sodium with equimolar amount of sucrose to maintain isosmolarity. The change of acetylcholine output has been expressed in percent taking the control output as 100. One experiment.

ganglion (Brown & Feldberg, 1936); and Liley (1956*a*) has shown a corresponding effect on rate of miniature discharge at the neuromuscular junction. Fig. 11*a* shows how resting output from the longitudinal strip varies with external potassium concentration (K_o). For small changes around the normal potassium content, there is little effect, but as concentration is raised, output increases dramatically. Liley (1956*a*) made similar observations and found that the rate of miniature discharge increased with the fourth power of K_o. A corresponding analysis of our results requires a preliminary correction. Evidence has been given earlier

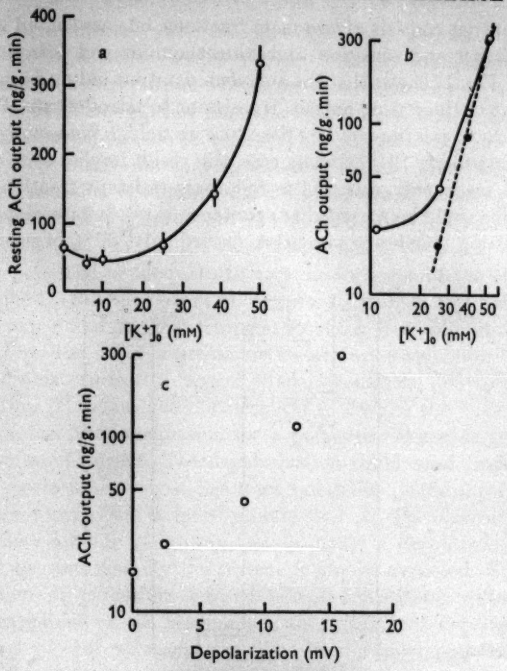

Fig. 11. Relation between acetylcholine release, external potassium concentration (K_o^+) and estimated depolarization of nerve terminals of eserinized longitudinal muscle strips of guinea-pig ileum. (*a*) Relation between acetylcholine release and K_o^+. To maintain isosmolarity, NaCl concentration of Krebs was lowered to the same extent as the KCl concentration was raised. Mean of six experiments; S.E. are indicated. (*b*) Correlation between K_o^+ and acetylcholine resting release. Data are plotted on double log scales. The tetrodotoxin-sensitive component of the control resting release of acetylcholine in 5·8 mM-K_o^+ has been subtracted from the outputs observed at the increased K_o^+ concentrations. The slope of the line from 27 mM-K_o upwards is 3·28. If the potassium-insensitive component of output (50·0 ng/g.min) is subtracted, the dashed line of potassium-sensitive release is obtained, with slope of 4·2. (*c*) Correlation between the depolarization produced by K_o^+, estimated by re-plotting the results of Armett & Ritchie (1963), and acetylcholine output induced by different concentrations of K_o^+. Abscissae: potential (x) is expressed relative to the resting potential obtaining at the normal potassium concentration (see Armett & Ritchie, 1963): the acetylcholine output (y) at different K_o^+ concentrations is plotted on a logarithmic scale. The tetrodotoxin-sensitive part of resting release has been subtracted from the values of acetylcholine output. The equation is $\log y = 0{\cdot}113x + 0{\cdot}6838$.

that the output consists of two main fractions, one associated with propagated activity and sensitive to hexamethonium and tetrodotoxin, the other not. Fig. 12 shows that the augmented output induced by potassium, unlike part of the resting output, is resistant to tetrodotoxin. Presumably nerve conduction is impaired by the exposure to high potassium concentration (cf. Lundberg, 1948). In any case, this result implies that comparison should be made only with the tetrodotoxin-resistant fraction of normal output; this could be regarded as spontaneous release associated with the normal resting membrane potential. Accordingly, 60% of normal resting

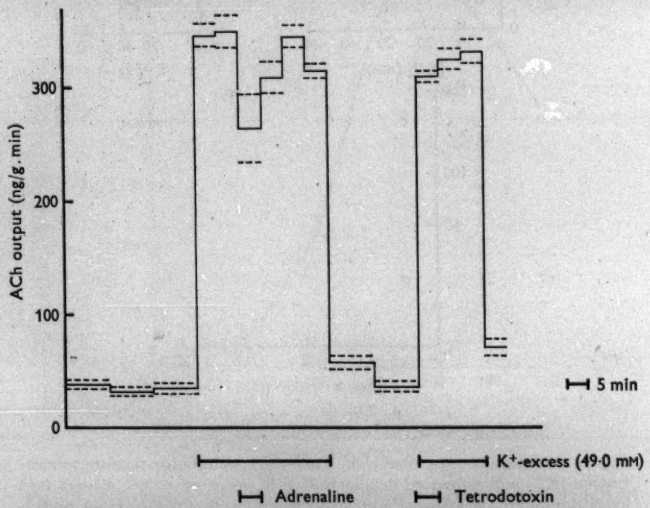

Fig. 12. The resistance to tetrodotoxin of acetylcholine output evoked by high K_o^+. Longitudinal muscle strip of guinea-pig ileum. Potassium, 49·0 mM, was present as indicated. Adrenaline, 10^{-6} g/ml., is present as indicated. Three experiments were made using identical dose and time schedules. Dashed lines represent the S.E. of the mean.

output has been subtracted from the acetylcholine outputs determined. Fig. 11*b* shows the relation of these outputs to K_o, plotted with logarithmic scales. With high K_o the output increases with a power of K_o of about 4. If assumptions are made as to the permeability of the nerve-ending membrane, estimates of the connexion between membrane potential and output could be made; but on comparing our results with those of Liley, it must be supposed that the region where the membrane potential is potassium-determined is only from 20 mM or beyond. A more direct approach is available, from the data of Armett & Ritchie (1963) on

the change of membrane potential, measured by a sucrose-gap method, with K_o, for desheathed vagus nerve C fibres. Fig. 11*c* gives the corrected acetylcholine output plotted against membrane depolarization, calculated on the assumption that the nerve fibres of the longitudinal muscle plexus correspond to vagus nerve C fibres in their response to change in K_o. The slope of the relationship varies so that for lower values of K_o, 15 mV depolarization is required for a tenfold rise in output (or a compound increase of 15 % per mV), but for high values of K_o only about 10 mV.

It is evident that the sensitivity of acetylcholine output to changes in membrane potential is adequate to account for some of the changes we have described. Thus the output in response to ouabain and PHMB could readily be attributed to depolarization resulting from sodium entry and potassium loss. The effect of potassium withdrawal is less obvious, but it is known that this can lead to spasm of smooth muscle, and to membrane depolarization (Holman, 1958; Paton, 1961), despite the expectation on the basis of the Nernst equation for a potassium electrode that hyperpolarization would occur. The depolarization could arise from a fall in potassium permeability as a result of potassium withdrawal, allowing other cation movements a more prominent role. There is no evidence, however, that sodium withdrawal should cause a depolarization of the nerve endings, and possibly the reverse might be expected. Nor can the augmentation of potassium-induced release by sodium withdrawal be plausibly explained on the basis of a further membrane depolarization.

The role of calcium and magnesium. The discrepancy mentioned could be accounted for, and a mechanism provided for the connexion between depolarization and release, if it were entry of calcium in the membrane that is critical for acetylcholine release, and if sodium competes with it in the way described for heart muscle and other tissues (Luttgau & Niedergerke, 1958; Colomo & Rahamimoff, 1968; Douglas & Rubin, 1963). It would then only be necessary to suppose that depolarization induces calcium entry and that sodium removal also increases calcium influx, as is known for squid axon (Baker & Blaustein, 1968; Hodgkin & Keynes, 1957). There is, indeed, abundant evidence that acetylcholine release in autonomic ganglia is normally calcium-dependent, since the pioneer work of Harvey & MacIntosh (1940). With the longitudinal strip, we have found that calcium is likewise normally essential for acetylcholine output (Fig. 13). In addition Ba^{2+} is found to substitute for it, giving a greatly enhanced output in the period immediately after substitution. Similar results have been obtained on the superior cervical ganglion by Douglas, Lywood & Straub (1961) and on m.e.p.p.s (Elmqvist & Feldman, 1965*a*). Further, magnesium is known to antagonize many actions of calcium (Hubbard, 1961; Skou, 1960) and would therefore be expected to interfere with acetylcholine output. A

fivefold increase of Mg^{2+} in the bath fluid from 1·2 to 6·0 mM reduced the resting acetylcholine output from 45·7 ± 2·0 to 17·4 ± 1·6 ng/g.min (n = 3). A similar finding has been made by Cowie, Kosterlitz & Watt (1968) who found that resting acetylcholine output from the longitudinal strip exposed to 0·1 mM-Mg^{2+} was roughly 10 times greater than with 5 mM-Mg^{2+}.

It would be expected on this basis, however, that all acetylcholine release should be calcium-dependent. But we have found that release of acetylcholine from the longitudinal strip caused by sodium deprivation

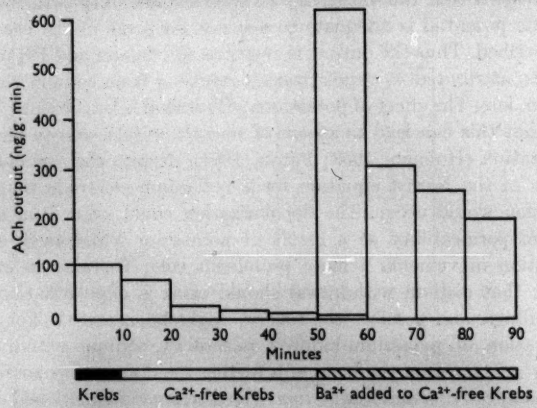

Fig. 13. Effect of Ca^{2+} lack and its substitution by Ba^{2+} on resting acetylcholine output. Mean of two experiments, which differ by less than 10 % at each value. Calcium-free solution was prepared by omitting calcium chloride from the bathing solution. Barium-Krebs was prepared by replacing $CaCl_2$ with $BaCl_2$. For method of barium extraction from the Krebs assay, see Methods.

does not depend on the presence of calcium (Fig. 14). MacIntosh (1963) has already reported that the same is true for the superior cervical ganglion.

It is thus possible to find conditions where enhanced acetylcholine release can take place without dependence on movements of sodium, nor on nerve terminal depolarization, nor on the presence of calcium in the extracellular fluid. This result, particularly the finding that deprivation of both sodium and calcium led to an augmented rate of acetylcholine release from the strip, prompted us to consider another theory (Zar, 1965): that the release of acetylcholine depends on the inhibition of ($Na^+ + K^+ + Mg^{2+}$)-activated ATPase associated with the membrane of the nerve endings. For on this hypothesis, once the ATPase has been inactivated through the absence of sodium, the presence of calcium would no longer be required for

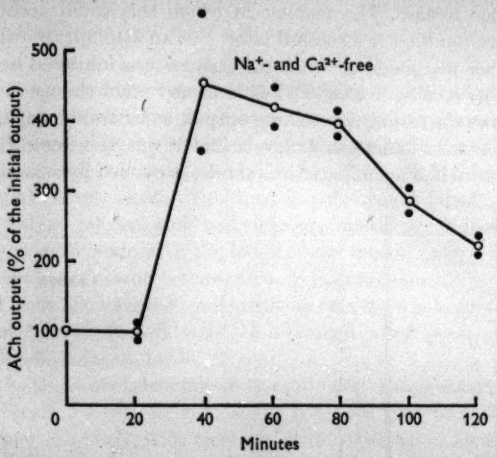

Fig. 14. Increase of acetylcholine output from the longitudinal muscle strip of guinea-pig ileum as a result of withdrawing sodium and calcium. Sucrose was used as the substitute for sodium and $CaCl_2$ was omitted. The value of acetylcholine output (○) represents the mean of two experiments. The individual values are indicated (●). The acetylcholine output in normal Krebs was taken as 100 and the subsequent values obtained have been expressed as % of the initial output.

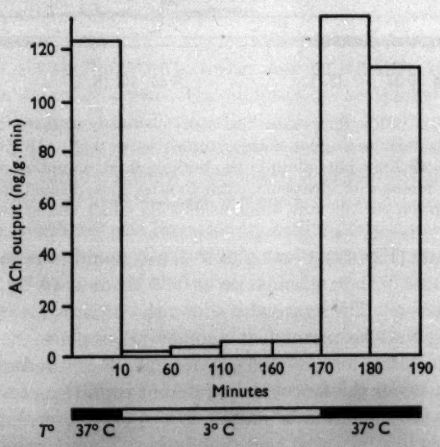

Fig. 15. The effect of lowering the temperature on the acetylcholine output from the longitudinal muscle strip of guinea-pig ileum. The acetylcholine output is expressed in ng/g.min. Mean of two experiments.

acetylcholine release. The manner in which this could account for our experimental findings is discussed later. But an attempt to test this hypothesis further was made in which the enzyme was inhibited not by added drugs, but by cooling to near 0° C without any other change in conditions. Fig. 15 shows the result, that resting output, so far from being increased, is reduced to almost undetectable levels. But it was also found that this fall of temperature had a similar effect on release evoked by stimulation or by

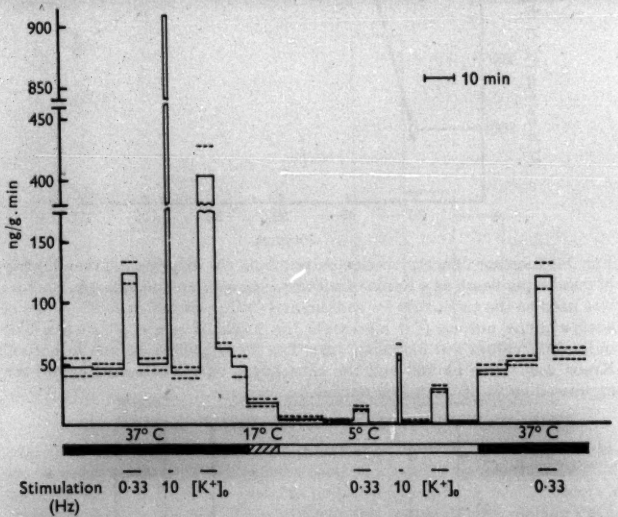

Fig. 16. Effect of low temperature on acetylcholine output by longitudinal muscle strip under resting conditions, stimulation at 0·33 or 10 Hz, or during exposure to 49·7 mM potassium in the bathing fluid, as indicated. Mean of three experiments with identical treatment schedules; dashed lines indicate S.E. Stimulation periods were 5 min at 0·33 Hz and 1 min at 10 Hz.

added potassium (Fig. 16). Cooling to 5° C was found to reduce by 90% or more the response both to stimulation at 0·33 Hz or at 10 Hz, and also that to 50 mM potassium. The figure also shows the prompt reversibility of the effect, showing that no permanent damage takes place. It was thus not possible to use cooling for a crucial experiment, since it reduced the acetylcholine output under the four widely different conditions tested. It should be noted that the reduction of output in response to cooling is probably not attributable to a failure of nerve conduction, since Greengard & Straub (1958) found that conduction in rabbit sympathetic fibres survived cooling to 7° C, the negative after-potential being greatly increased.

Acetylcholine synthesis by the longitudinal muscle strip

It is known from work with cat superior cervical ganglion that although 85% of the acetylcholine content of the ganglion is available for release, yet under normal conditions the content is maintained remarkably constant when the ganglion is stimulated even though more than the original content has been released (Birks & MacIntosh, 1961). As MacIntosh (1963) particularly has emphasized, there must be some control mechanism linking acetylcholine release and acetylcholine synthesis. Experiments of a similar type were made with the longitudinal muscle strip, in which the effect of a number of the procedures already discussed on acetylcholine was determined, thus allowing the total synthesis rate to be calculated (Table 2) The control figures quoted were all obtained after pre-incubation in eserinized Krebs solution for 60–90 min.

At rest, the tissue acetylcholine content did not change significantly, the rate of synthesis, $3{\cdot}4 \pm 0{\cdot}5$ μg/g.hr, being sufficient fully to make good the resting output. Similarly, with electrical stimulation at 10 Hz or exposure to raised external potassium concentration (49·0 mM), synthesis rose to a level, $19{\cdot}8 \pm 1{\cdot}3$ and $20{\cdot}3 \pm 0{\cdot}6$ μg/g.hr respectively, sufficient or almost sufficient to compensate for release. It is possible that during stimulation synthesis lagged a little behind release. With barium treatment, in addition, synthesis and release were closely matched. The fact that these three procedures each induced a synthesis rate of about 20 μg/g.hr, together with the fact that acetylcholine output per minute does not increase with higher rates of stimulation than 10 Hz, suggests that this represents the maximal synthesis rate. At the opposite extreme may be cited the experiments in which calcium was withdrawn. The abolition of acetylcholine release by this procedure has been already described; the acetylcholine content of the tissue fell, but the change in these experiments was of doubtful significance. Although more evidence is required, it appears that with calcium lack, also, release and synthesis are matched.

In contrast, synthesis was impaired by sodium or potassium lack or by ouabain treatment, although acetylcholine release was increased. The effect of sodium deprivation was the most striking, synthesis being reduced below the resting level, and possibly being arrested altogether. With ouabain and potassium lack, synthesis did not rise above the resting level. This impairment of synthesis probably affects the estimate of the acetylcholine-releasing capacity of these procedures, since it would be expected that the release would have been bigger had the tissue not become depleted of acetylcholine. The depletion may also contribute to the falling away of the rate of release with time; but since the release induced by raised potassium concentration also falls away with time, other factors

TABLE 2. Effect of various procedures on acetylcholine content of and acetylcholine release from nerve terminals of eserinized longitudinal muscle strips from guinea-pig ileum and on calculated rates of acetylcholine synthesis

Condition	n	ACh content (μg/g ± S.E.) before	ACh content (μg/g ± S.E.) after	Gain (+) or loss (−) (μg/g ± S.E.)	Total release (μg/g ± S.E.)	Total synthesis (μg/g ± S.E.)	Rate of synthesis (μg/g.h ± S.E.)
1. Resting (1 hr)	3	21·8 ± 1·6	22·5 ± 1·4	+0·7 ± 0·3	2·6 ± 0·2	3·4 ± 0·5	3·4 ± 0·5
2. K^+ excess (49 mM) (20 min)	3	23·4 ± 0·6	25·7 ± 1·3	+2·3 ± 0·7	6·0 ± 0·2	8·3 ± 0·6	20·3 ± 0·6*
3. Stimulation 12/min (3 hr; no eserine)		20·4 ± 3·7 (4)	17·7 ± 2·1 (5)	−2·7	36 (estimated)	34·3	11·4***
4. Stimulation 10 Hz (1 hr)	5	22·2 ± 0·6	17·5 ± 0·7	−4·7 ± 1·1	25·2 ± 0·7	19·8 ± 1·3	19·8 ± 1·3
5. K^+ lack (2 hr)	2	25·8 (2)	8·4 (2)	−17·4	26·1	8·7	4·4**
6. Na^+ lack (2 hr)	2	28·2 (2)	2·5 (2)	−25·7	26·9	1·2	0·6**
7. Ouabain (1 hr)	5	23·5 ± 0·6	17·2 ± 0·8	−6·1 ± 0·4	11·0 ± 0·9	4·8 ± 0·7	4·8 ± 0·7
8. Ca^{2+} lack (2 hr)	2	21·1	17·5	−3·5	2·5 (estimated)	(−1·0)	(−0·5)
		34	25·3	−8·7	2·5 (estimated)	(−6·2)	(−3·1)
9. Ba^{2+} treatment (69 min; includes 2 min stimulation at 10/sec)	2	25·9 (2)	26·0 (2)	+0·1	26·0	26·1	22·7

The solutions and conditions used were: (1) resting (Krebs solution); (2) K^+ excess (49·0 mM-KCl with NaCl lowered to maintain isosmolarity); (3) stimulation 0·2 Hz (Krebs solution, no eserine present); (4) stimulation 10 Hz for 1 hr (Krebs solution); (5) K^+ lack; (6) Na^+ lack; (7) Ouabain 5×10^{-5} M; (8) Ca^{2+}-free; (9) Ba^{2+}.

* Calculated for 1 hr from 20 min exposures; ** Calculated for 1 hr from 2 hr exposures; *** calculated for 1 hr from 3 hr exposures. Values are given of mean ± S.E. (except (5), (6), (8) and (9) when values or mean of two experiments is cited. n refers to the number of experiments.

such as depletion of the readily mobilized stores (Birks & MacIntosh, 1961; Paton & Vizi, 1969; Collier & MacIntosh, 1969) may be important. It was interesting that in the sodium withdrawal experiment, an appreciable output was still taking place when acetylcholine content must have been drastically reduced.

DISCUSSION

Most of our recent knowledge concerning acetylcholine release has been derived from experiments on nerve-striated muscle preparations (Fatt & Katz, 1952; Katz, 1962; Liley, 1956*a*, *b*; del Castillo & Katz, 1954; Katz & Miledi, 1965, 1967) or the cat superior cervical ganglion (Brown & Feldberg, 1936; Perry, 1953; Birks & MacIntosh, 1961; Birks, 1963; MacIntosh, 1963). The longitudinal muscle strip preparation of guinea-pig ileum provides information about acetylcholine release at a different type of neuroeffector junction. It possesses the advantages that relatively large amounts of acetylcholine are released; it is now established that the acetylcholine comes only from the nervous tissue (Paton & Zar, 1968); and the preeffector cholinergic axones can be assumed still to be in continuity with their cell bodies.

It would be expected that acetylcholine is released by a process corresponding to the quantal release at motor end-plate or ganglionic synapse. Although there is no direct evidence, such as miniature junction potentials, for quantal release in this preparation, vesicles with a diameter ranging from 250 to 600 Å are found in the terminal nerve bundles of the plexus (Fig. 17). Since close approximation of nerve terminal to smooth muscle cell is not common (W. D. M. Paton, unpublished), discrete junction potentials may in fact not be readily detectable. At the same time, since release from the nerve terminals of the longitudinal muscle strip is far more sensitive to drugs such as morphine or noradrenaline than is the case at motor end-plate or ganglion, the possibility of some other process of release must be kept in mind; in view of the presence of cholinesterase in the membrane of cholinergic nerves, however, it is difficult to envisage a physiologically plausible mechanism.

The principal disadvantage of the preparation is that the state of activity of the nervous tissue is less well defined than for the other synapses. It was concluded, from experiments with tetrodotoxin and hexamethonium, that in the resting state (i.e. without external electrical stimulation) about 60% of the resting acetylcholine output depended on propagated activity. A similar partition of miniature junction potential discharge into tetrodotoxin-sensitive and tetrodotoxin-resistant has been made at the neuromuscular junction, and the latter is usually held responsible for the acetylcholine output in the absence of stimulation (Katz & Miledi, 1967;

Elmquist & Feldman, 1965*b*). It is not yet clear how far the tetrodotoxin-resistant part of acetylcholine release from neuromuscular junction can properly be compared with that from longitudinal muscle strip, but at least it is now known that both can be increased by potassium.

We have described above how the theories that acetylcholine release is ultimately controlled by axonal sodium concentration, by membrane potential, or by calcium failed to account for all our results. The fact that

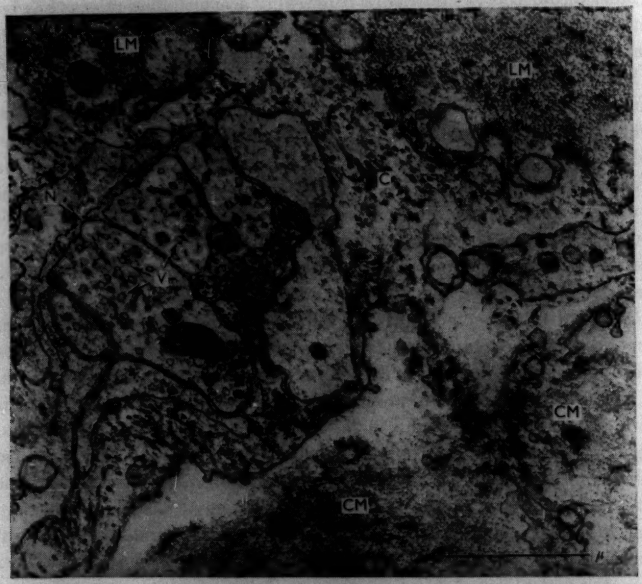

Fig. 17. Electron micrograph of section of longitudinal muscle strip showing a small nerve bundle (N) between longitudinal smooth muscle cells (LM) cut transversely and circular smooth muscle cells (CM) cut longitudinally. Osmium fixation, Araldite embedding and lead citrate after-staining. Collagen bundles (C) usually cut transversely lie between the nerve bundle and the muscle fibres. Vesicles (V) are present in the axons of the nerve bundle. Bar 1 μm.

acetylcholine release as a result of sodium deprivation did not require the presence of calcium suggested that release might depend on inhibition of the activity of a $(Na^+ + K^+ + Mg^{2+})$-activated ATPase at the axonal membrane. This enzyme has been shown to be present in the membrane of nervous tissues (Skou, 1957; Bonting, Caravaggio & Hawkins, 1962; Somogyi, 1964) as well as in the erythrocyte and cardiac muscle membrane

(Dunham & Glynn, 1961; Repke, 1965). It remains to consider how far such a mechanism could account for our results generally.

(*a*) The activity of magnesium-activated ATPase of crab nerve is almost abolished by sodium deprivation (Skou, 1957). Whittam (1962) has shown that the membrane ATPase of the erythrocyte membrane has vectorial properties, so that it is probable that the inhibition would develop only slowly in experiments on whole tissues, as the sodium was lost from the interior of the axon. Lithium has been found to be a far less effective activator of the enzyme than is sodium; it competes with sodium (Skou, 1965) and in its presence the intraneuronal sodium concentration falls (Wespi, 1969; Baker, Blaustein, Hodgkin & Steinhardt, 1969). Substitution of sodium by lithium could, therefore, lead to a more rapid functional sodium deprivation and hence ATPase inactivation. Acetylcholine release developed more rapidly when sodium replacement was with lithium rather than with Tris or sucrose (Fig. 4). Acetylcholine release by such a mechanism would not be expected to be calcium-dependent.

(*b*) The same enzyme has also been shown to be dependent on potassium, and very low levels of activity prevail in its absence (Skou, 1957).

(*c*) Ouabain was one of the first inhibitors of the enzyme to be identified (Skou, 1957; Bonting *et al.* 1962), and it is now known to be active against ATPase from brain, red cells, striated muscle, cardiac muscle and mucous membrane (Bonting *et al.* 1962; Dunham & Glynn, 1961; Repke, 1965; Smith, 1967). Maximal inhibition may require about 10^{-4} M ouabain (Repke, 1965), but half-maximal inhibition of enzyme preparations is usually obtained with about $0{\cdot}1$–1×10^{-6} M (Smith, 1967; Bonting *et al.* 1962). These results have been obtained on tissue homogenates in which the glycoside would have ready access to the enzyme. In our experiments, an effect was obvious with 10^{-6} g/ml. ($1{\cdot}4 \times 10^{-6}$ M) and considerably greater with $3{\cdot}7 \times 10^{-5}$ g/ml. (5×10^{-5} M). The concentrations of ouabain needed to produce an effect appear high compared to those required for ATPase inhibition; but this could be due partly to restraints on the access of the glycoside to the enzyme in intact unhomogenized tissue, and partly to a simultaneous release of catecholamine (Banks, 1970), which is known to reduce acetylcholine output (Paton & Vizi, 1969; Kosterlitz, Lyden & Watt, 1970; Knoll & Vizi, 1970). The experiments of Elmquist & Feldman (1965*a*) raise a question about ouabain action. They found that although ouabain continued to cause an increased discharge of m.e.p.p.s in the rat diaphragm in the absence of calcium, even if EDTA was added at the same time as the ouabain, ouabain failed to be effective if the tissue was incubated previously in calcium-free EDTA-containing medium. They suggested, therefore, that ouabain works in part by mobilizing sequestered calcium; on other grounds, they concluded that its action was also due in

part to inhibition of the sodium pump. In either case, however, ATPase inhibition would be expected.

(*d*) PHMB has been shown to inhibit the ATPase of ox brain (Skou, 1963); in a concentration of 10^{-5} M, 60% inhibition was produced. It is, however, an inhibitor of less specificity than the cardiac glycosides, being a general sulphydryl reagent.

(*e*) It has long been known that acetylcholine release does not take place in the absence of calcium, and there is now convincing evidence that release as a result of a change in membrane potential of the nerve ending, produced by a propagated action potential, by local depolarization, or by added potassium, depend on the presence of calcium and its interaction with or entry into the membrane. Calcium is also a very effective inhibitor of ATPase; Skou (1957) found that 50% inhibition is produced by about 0·5–1 mM, in the presence of 120 mM potassium, 80 mM sodium and 3 mM magnesium; the inhibition is sensitive to the concentration of other ions, and magnesium in particular antagonizes its action (Skou, 1957, 1960; Rendi & Uhr, 1964). Somogyi (1964) showed, with membrane ATPase from rat brain, that the concentration of Ca^{2+} to produce half-maximal inhibition was 0·5 mM, in the presence of 100 mM sodium, 20 mM potassium and 5 mM magnesium. In our experiments, therefore, the output evoked by stimulation or by K^+ excess could be due to Ca^{2+} movement with resultant ATPase inhibition. The ability of Mg^{2+} to reduce acetylcholine output could, conversely, be attributed to ATPase activation (Skou, 1957, 1960).

(*f*) Barium is known to be able to substitute for calcium in transmitter release (Douglas *et al.* 1961). In a concentration of 10^{-6} M it inhibits ATPase of calf kidney by 100% (Rendi & Uhr, 1964). Elmqvist & Feldman (1965*a*) showed that barium increases m.e.p.p. discharge, and can do so when calcium is removed with EDTA.

Our results can, therefore, be accounted for qualitatively in an economical way on the assumption that acetylcholine release depends on inhibition of $(Na^+ + K^+ + Mg^{2+})$-activated ATPase in the nerve terminal membrane. But consideration of the data of Table 2 raises another question. A crucial experiment was the observation that release occurred in response to simultaneous sodium and calcium deprivation. In Table 2 it is shown that sodium deprivation leads to a virtual suppression of synthesis, and a similar observation has been made by Birks (1963) on the cat superior cervical ganglion. It could be suggested therefore that the increased rate of release found in the absence of sodium is in some way linked to this suppression of synthesis, perhaps through interference with acetylcholine storage sites (cf. MacIntosh, 1963), and that this entails a release process distinct from that normally involved. The output resulting from the other procedures used could in turn be plausibly linked to calcium mobilization

or influx. There is, however, no reason to suppose that interference either with storage or with synthesis of acetylcholine by sodium deprivation will lead to an *increased* rate of release. If impairment of acetylcholine storage by sodium deprivation led to the appearance of free acetylcholine within the nerve ending, the situation would be analogous to that of 'surplus' acetylcholine in the superior cervical ganglion. Surplus acetylcholine was the term applied by Birks & MacIntosh to the increase in content that follows treatment of the superior cervical ganglion with eserine, an increase sufficient to double the acetylcholine content of the ganglion. They concluded that it was probably acetylcholine surplus to that required for vesicular acetylcholine, normally destroyed by cholinesterase, but preserved in the axoplasm in the presence of eserine. They found, however, that in the presence of this surplus acetylcholine, neither resting output nor output in response to preganglionic stimulation was increased and they concluded that it was not released during normal transmission. A possibly analogous finding is the observation by Potter (1967) that although in the presence of reserpine and an amine oxidase inhibitor a sympathetic nerve may still contain noradrenaline, free in the axoplasm, it cannot be released by nerve stimulation. Nor does the mere inhibition of synthesis entail release of acetylcholine; HC3 in suitable doses virtually abolishes synthesis, but does not itself lead to acetylcholine release (MacIntosh, 1963).

The suggestion that ATPase inhibition is necessary for acetylcholine release might imply that ATPase activity generally is necessary for stability of the nerve terminal membrane; and that only regions of instability can be used for exocytosis. Alternatively, there might be a more specific link with the ATPase-dependent transport mechanisms such that only inhibited channels were available for use in the process of exocytosis.

The control of synthesis of acetylcholine by the nerve plexus remains puzzling. MacIntosh (1963) has stressed the way in which the superior cervical ganglion maintains a remarkably constant acetylcholine content under varying conditions of synaptic activity, the rate of synthesis being in some way adjusted both to the activity and to the capacity of the stores. The longitudinal muscle strip behaves in the same way, at rest, or when stimulated electrically, or by potassium, or with barium. With widely varying rates of release, acetylcholine content is held rather constant. It is difficult, however, to argue that sodium entry controls this homoeostatic process, since treatments with ouabain or with potassium-deprivation which lead to sodium entry (Hodgkin & Keynes, 1955) produce depletion of the tissue acetylcholine content, the synthesis rate being only about sufficient to compensate for normal resting output. It is equally doubtful whether calcium entry could be held to control the process, since ouabain is known to increase calcium uptake of cortex (Stahl & Swanson, 1969), yet

the synthesis when exposed to ouabain is not increased significantly above the resting level; sodium deprivation also leads to increased calcium influx (Baker & Blaustein, 1968), yet here, as already discussed, synthesis is almost arrested. It is, however, suggestive that there are three procedures, namely treatment with ouabain, withdrawal of sodium and withdrawal of potassium, which both impair synthesis and promote release.

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Volume 215

1971

CAMBRIDGE UNIVERSITY PRESS

PUBLISHED BY
THE SYNDICS OF THE CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London NW1 2DB
American Branch: 32 East 57th Street, New York, N.Y. 10022

Printed in Great Britain at the University Printing House, Cambridge

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THE JOURNAL OF PHYSIOLOGY, JULY 1971

VOL. 215. No. 3

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Cambridge University Press, 200 Euston Road, London NW1 2DB

American Branch: 32 East 57th Street, New York, N.Y.10022

Price £2.50 net ($8.00 in U.S.A.) Annual subscription £48.00 net ($156.00 in U.S.A.)

Printed in Great Britain at the University Printing House, Cambridge

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